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(54) Title: COMPOSITION COMPRISING IMMUNOGENIC MICROPARTICLES

(57) Abstract: The invention provides an immunogenic composition comprising at least one antigen in association with microparticles, wherein the microparticles are in the same size range as viruses. In addition the invention also provides vaccine compositions and methods of eliciting immune responses in a subject.

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COMPOSITION COMPRISING IMMUNOGENIC MICROPARTICLES

The present invention relates to immunogenic compositions, vaccine compositions, methods of eliciting immune responses in a subject and methods of producing the compositions.

Background of the Invention

Manipulation of the immune systems of humans and animals is a recognised manner of avoiding or treating certain diseases or conditions.

The mechanisms by which the immune system controls disease include the induction of neutralising antibodies (a humoral immune response), and the generation of cellular or T-cell responses. The latter include T-helper cells (T_H) and cytotoxic T-lymphocytes (CTL). In instances of viral infection e.g. polio or hepatitis, antibodies provide protection by preventing the virus from infecting cells. Antibodies can also protect against bacteria e.g. pneumococci and staphylococci, by use of bactericidal mechanisms and by neutralising bacterial toxins.

T-cells can be stimulated when peptide fragments from an antigen are bound to molecules known as MHC I or MHC II (major histocompatability complex, class I or class II) and are displayed on the surface of professional APCs (antigen presenting cells) such as DCs (dendritic cells) or macrophages. The T-cells contain antigen receptors which they employ to monitor the surface of cells for the presence of the peptide fragments from the antigen. The antigen receptors on T_H cells recognise antigenic peptides bound to MHC II molecules. By contrast, the receptors on CTLs react with antigens displayed on class I molecules.

The stimulated T-cells amplify the immune response in that when a T-cell recognises a target cell which is infected with the pathogen, or that contain an epitope which it recognises, a chain of events is triggered and these eventually result in death of the infected

cells. In addition, some T-cells can stimulate secretion of cytokines or lymphokines, which in turn can exert effects that ultimately lead to inactivation or eradication of pathogens.

Although there are many vaccines on the market there is a need to produce more effective and broad ranging vaccines for a number of diseases or conditions. There also remains a need for protection against infective agents or pathogens against which vaccines are currently unavailable or ineffective. In addition, there is a need for effective, single-dose vaccines, which are particularly desirable for economic reasons, for ease of delivery, and for patient or subject compliance.

Most vaccines suffer from the disadvantage that they are not able to induce an optimal combination of the various types of humoral and cellular responses so as to be immunologically effective. For instance, some vaccines only stimulate antibody responses when both antibody and cellular responses would be more efficacious. In other instances, multiple doses of the vaccines eg booster shots are required in order to attain protection against the relevant infective agent.

In some other instances, IgE production is induced along with other desired immunoglobulins such as IgA, IgG and IgM. Vaccines that induce IgE are not desirable, as the immunoglobulin is involved in allergic responses.

Stimulation of IgA production is a "first line" defence for pathogens that infect via entry through a mucosal site or surface. Thus, vaccines that can generate a high IgA secretory immune response without enhancing IgE production would also be valuable.

In yet other instances, although a vaccine results in stimulation of APCs, the degree of immune stimulation is sub-optimal. For example, dendritic cells or DCs are characterised by a series of subset of cells that can be distinguished from each other by surface molecules some of which are specific ligands that bind receptors on T cells. Accordingly, it would be desirable to produce a vaccine which would selectively target

a subset of DCs, eg a subset capable of efficient CD8 T-cell priming since these T-cells play a vital role in protective immunity against many intracellular pathogens and cancer, but are notoriously difficult to induce.

Further, with regard to vaccines extracellular antigens traditionally do not enter the MHC-I processing pathway in most cells. In general, the production of CTL immunity using nonliving vaccines is unlikely although alternative routes of processing and presentation for class 1 have been proposed in APC through the uptake of apoptotic cells, immune complexes and particles [1]. Non-infective viral like particles (VLP) composed of the surface Hepatitis B protein or yeast retro-transposon protein particles have been shown to be efficiently processed for MHC I presentation by macrophages to induce CD8 CTL responses *in vitro* and *in vivo* [2, 3]. VLPs are multimeric, lipid-containing protein particles the lipid content of which comprises more than 50% of the dry weight.

However, since Hepatitis B core protein particles fail to be immunogenic, and have a lower lipid content, it has been proposed that VLP are immunogenic not by virtue of size, but by biochemical composition. This would be consistent with the proposal that when antigen is presented in formulations containing lipid or detergent, they are able to fuse with the APC, possibly by damaging the cell membrane, and thus gain entry into the cytoplasm.

The use of microspheres within which are entrapped antigens have been explored as a possible vaccine composition. The microspheres are made from biodegradable polyesters of lactic and glycolic acids (PLA and PLGA). The microspheres are constructed such that their size and polymer composition control the rate at which they degrade. As the microspheres degrade, the entrapped antigen is released therefrom, and provides for a controlled release of antigen for stimulating the immune response. It is unlikely that these molecules would interface and react with immune cells in the same way as protein

particles the make-up of which are biologically compatible with cellular membranes.

However, the difficulties with this form of vaccine composition include antigen stability, the size of the spheres and the antigen-release kinetics, all of which still need to be resolved so as to produce a vaccine with good antigenicity and lasting immunogenicity, and to produce a vaccine that can be manufactured and administered economically [4].

In US Patent no. 4,225,581, a composition comprising a mixture of heterogenous particles ranging in size is described as being useful for delivering antigens that are adsorbed onto the surface of the polymeric particles to the body. However, the successful delivery, antigenicity and immunogenicity of such a vaccine was not illustrated or shown. Specifically, there was no reference to the induction of CD8 T cell responses, or even processing into the MHC class I presentation pathway. The polymeric material of the particles would be expected to have similar characteristics as PLA or PLGA microparticles discussed above.

Thus, it was not known prior to the present invention if the size *per se* of particles administered as part of vaccines could induce immunogenic responses.

In work leading up to the present invention, the inventor has surprisingly found that microparticles about the same size as viruses associated with an antigen provide strong cellular and humoral antibody responses in subjects.

Summary of the Invention

In a first embodiment the invention provides an immunogenic composition comprising at least one antigen in association with microparticles wherein the microparticles are in the same size range as viruses.

The term "comprising" used in relation to the immunogenic composition means that the composition includes the antigen and microparticles. It may also include other components.

The term "antigen" refers to any molecule, moiety or entity capable of eliciting an immune response. This includes cellular and/or humoral immune responses. Depending on the intended function of the composition one or more antigens may be included.

The antigen may be a peptide, protein, lipid, carbohydrate, nucleic acid or other type of molecule or a combination of any of these.

The antigen may be derived from a pathogen, tissue, cell, organ or molecule depending on the intended purpose of the composition, and may be a purified antigen, or be of recombinant origin produced in suitable vectors such as bacteria, yeast or cell cultures. The pathogen for example may be any pathogen, intra or extracellular, antigenic portions or parts thereof, viral, bacterial or protozoal in origin such as HIV, influenza viruses, hepatitis viruses, malaria. Specifically, examples of the antigens envisaged by the present invention are as follows: pollens, hepatitis C virus, (HIV) core, E1, E2 and NS2 proteins, antigens from Plasmodium species such as P. vivax and other Plasmodium species including P. falciparum circumsporozoite protein (CS) and human Plasmodium-falciparum, -vivax, -ovalae and malariae, TRAP, MSP-1, MSP-2, MSP-3, MSP-4, MSP-5, AMA-1, RESA, SALSA, STARP, LSA1 and LSA3, HIV-gp120/160 envelope glycoprotein, streptococcus surface protein Ag, influenza nucleoprotein, haemagglutinin-neuraminidase surface infection, TcpA pilin subunit, VP1 protein. LMCV nucleoprotein, Leishmania major surface glycoprotein (gp63), Bordetella pertussis surface protein, rabies virus G protein, Streptococcus M protein, Staphylococcal proteins or Helicobacter pylori proteins, Syncyticial virus (RSV) F or G proteins, Epstein Barr virus (EBV) gp340 or nucleoantigen 3A, haemagglutinin, Borrelia burgdorferi outer surface protein (Osp) A, Mycobacterium tuberculosis 38kD lipoprotein or 30kD protein (Ag85), 10kD or 65kD

proteins, Neisseria meningitidis class 1 outer protein, Varicella zoster virus IE62 and gpl, Rubella virus capsid protein, Hepatitis B virus pre S1 ag, Herpes simplex virus type I glycoprotein G or gp D or CP27, Murray valley encephalitis virus E glycoprotein, Hepatitis A virus VP1, polio virus capsid protein VP1, VP2, VP3 and VP6, chlamydia trachomatis surface protein, Hepatitis B virus envelope Ag pre S2, Human rhinovirus (HRV) capsid, papillomavirus peptides from oncogene E6 and E7, Listeria surface protein, Varicella virus envelope protein, Vaccinia virus envelope protein, Brucella surface protein, Rotavirus VP-3, VP-4, VP-5, VP-7 and VP–8, a combination of one or more of said antigens, an amino acid subunit of said antigens comprising five or more amino acids in length or combinations of one or more of said subunits.

Lysates or culture filtrates from the pathogens exemplified above may also be used as the antigen. Such fractions may be in purified, concentrated or diluted form, so long as they provide antigenicity and/or immunogenicity. Thus it makes it possible to "tailor-make" an immunogenic composition for a patient in accordance with the invention by using patient tumor lysates or a specific set of tumor proteins conjugated to the microparticles.

The antigen may also be derived from any tumour type or malignancy. Examples of cancer types from which the antigens may be derived are breast, lung, pancreas and colon cancer and melanoma. Some further examples of specific antigens obtained from tumours are melanoma specific antigen (for example, the MAGE series antigen), carcino embryonic antigen (CEA) from colon, nm23 cancer antigen and other cancer antigens or indeed antigens extracted from any tumour, e.g. mucin such as MUC-1 to MUC-7 antigens. Recombinant peptides or proteins alone or in combination may also be used.

The antigen may also be a synthetic epitope such as a mimic or peptidomimetic based on one or more of the antigens referred to above.

The term "in association with" refers to an association between the microparticle and the antigen. This may be by adsorption or by

conjugation or covalent coupling. Preferably the antigen is covalently linked to the microparticle. Even more preferably, the antigen is conjugated to the surface of the microparticle.

The term microparticle refers to a small particle. This may be in the form of a bead or sphere or any other suitable shape.

The term "virus sized particles" (VSP) is used in this document to describe certain embodiments of the immunogenic composition of the invention. It should be understood that the term VSP has only been adopted for convenience and does not limit the invention to the size of known viruses. For Example, particles of the same size as unknown viruses are also contemplated by the invention.

Preferably the microparticle has a solid core providing stability to the conjugated or associated antigens as distinct from the microspheres of the prior art which are hollow or encapsulate molecules. For convenience these are referred to herein as virus sized solid particles (VSSP) where the antigen is present on the outside of the particle. The particles used to make VSSP are available from the manufacturer and are substantially of uniform size (i.e., within ±10% of the stated size).

The term "solid core" means substantially solid (i.e. the particles are not hollow). The microparticle may be composed of any suitable material so long as it does not detract from the function of the immunogenic composition. Thus the microparticles may be made from materials such as latex, ferrous molecules, gold (such as gold nanoparticles), glass, calcium phosphate, polystyrene or biodegradable and biocompatible polymers such as PLG (Polylysine g). Preferably, the microparticles are composed of polystyrene, PLG or gold. Most preferably, the microparticles are made from polystyrene.

The microparticle is in the same size range as known viruses. This means that the microparticle is preferably less than about 0.50μm. Preferably the microparticle is of such a size that it is adapted to elicit an immune response. In particular it is adapted to be taken up by antigen presenting cells within a human subject or an animal. More

preferably the microparticles are between about 0.03 and 0.50μm, preferably about 0.03 and 0.15μm, still more preferably between about 0.03 and 0.10μm. Even more preferably the microparticles are about 0.03 to 0.05μm, more preferably the microparticles are between about 0.03μm and 0.049μm, still more preferably the microparticles are between about 0.03 and about 0.04μm or about 0.04 and 0.049μm.

In a preferred embodiment, a population of microparticles to be used in accordance with the invention, eg in one dose of vaccination, is of a uniform size. This means that the majority of the particles in a given population are of the stated size.

Preferably the microparticle/antigen composition is particularly adapted to elicit a cellular and/or humoral immune response. The cellular response is preferably selected from the group consisting of activation, maturation or proliferation of T_H cells, in particular IFN and IL4 producing T cells, CTLs, particularly CD8 CTL and B cells. Preferably the microparticle/antigen composition elicits mechanisms for MHC class I presentation of antigens which are taken up by a hitherto unknown mechanism involving caveole and/or clathrin pits for further processing by Rab 4 independent and TAP dependent processes as explained in Examples 4 and 7 herein. The humoral response is preferably selected from the group consisting of IgA, IgD, IgG, IgM and subclasses thereof.

Cells which assist in mounting or amplifying an immune response may also be stimulated by the composition. These included but are not limited to APCs such as DCs of both myeloid or lymphoid origin, and macrophages. The maturation, activation or proliferation of such cells are contemplated, as are the co-stimulatory ligands or molecules on such cells that interact with T-cells, eg CD 40, CD 80 and CD 86.

The immunogenic composition of the invention may be used in treatment, prophylaxis or prevention of the disease or condition caused by, or associated with contact with the antigen. For example, the

composition may be used in the treatment or prophylaxis of certain cancers.

In another embodiment the invention provides a vaccine composition comprising microparticles associated with at least one antigen wherein the microparticles are in the same size range as viruses. The composition of the invention is particularly useful and advantageous as it is an effective single-dose vaccine but may also be used in multiple dose regimes.

Thus, in one embodiment, the invention provides a single-dose vaccine composition comprising microparticles associated with at least one antigen, wherein the microparticles are of the same size range as viruses.

By "single-dose", it is meant that a humoral and/or cellular immune response is stimulated or enhanced to maximal levels ("maximal" means that the levels are not capable of being further increased by repeated vaccination), or affords protection to the recipient of the composition, following one administration of the composition or vaccine. The administration may be by any suitable means eg. by injection i.m., i.p., i.v., orally, by inhalation, or by administration through a mucosal surface or site.

Preferably, the antigen is conjugated to the surface of the microparticles. The size of the microparticles is between about 0.03 and 0.5μm, preferably about 0.03 to 0.15μm, more preferably about 0.03 to 0.10μm even more preferably about 0.03 to 0.05μm in diameter, even more preferably about 0.03 to 0.049μm. Still more preferably 0.03 to 0.04μm or 0.04 to 0.049μm. The microparticles are most preferably made of polystyrene, PLG, glass, calcium phosphate or gold. In a preferred embodiment, each antigen for use in accordance with the invention is conjugated to microparticles of a uniform size.

In a further embodiment, the invention provides a single-dose vaccine composition that is capable of mounting a humoral and a cellular immune response, the composition comprising microparticles

associated with at least one antigen, wherein the microparticles are in the same size range as viruses.

The cellular response is preferably selected from the group consisting of stimulation, maturation or proliferation of T_H cells, CTLs and B cells. The humoral response is preferably selected from the group consisting of IgA, IgG, IgM and subclasses thereof. Preferably, IgG, IgA and/or IgM responses are stimulated.

Cells which assist in mounting or amplifying an immune response may also be stimulated by the composition. These included but are not limited to APCs such as DCs of both myeloid or lymphoid origin, and macrophages. The maturation, activation or proliferation of such cells are contemplated.

The term "comprising" has the same meaning given above.

The term microparticle has the meaning given above. Preferably the microparticle is adapted to be taken up by antigen presenting cells in an animal. Preferably the microparticles are between 0.03 and 0.5μm, preferably between 0.03 and 0.15μm. More preferably the microparticles are between about 0.03 and 0.10μm, more preferably the microparticles are between about 0.03μm and about 0.05μm. Still more preferably the microparticles are about 0.03 to 0.049 or 0.04 and 0.049μm.

The terms "antigen" and "associated with" have the meanings given above. Any suitable antigen may be used depending on which condition/disease it is intended to vaccinate against.

The amount of vaccine composition of the invention delivered to a patient is not critical or limiting. An effective amount of the vaccine composition is that which will stimulate an immune response against the antigen component, preferably after a single dose or administration and desirably, will result in strong cellular and humoral responses. The amount of compositions delivered may vary according to the immune status of the patient (depending on whether the patient is immunosuppressed or immunostimulated), the judgement of attending

physician or veterinarian, whether the composition is used as a vaccine to prevent or treat a disease state, or as a vaccine to prevent tumour formation, or whether the vaccine is used in the treatment of an existing tumour. By way of example, patients may receive from 1 μg to 10,000 μg of the composition of the invention, more preferably 50 μg to 5,000 μg, still more preferably 100 μg to 1,000 μg, and even more preferably 100 μg to 500 μg of the composition of the invention. Adjuvants are not generally required. However, adjuvants may be used for immunization. Suitable adjuvants include alum, as well as any other adjuvant or adjuvants well known in the vaccine art for administration to humans.

The vaccine of the invention may be administered by injection, by administration via the oral route, by inhalation or by administration via a mucosal surface or site. In one embodiment, the vaccine is administered by means of a gene gun. Ferrous microparticles and gold microparticles if used in accordance with the invention are especially suitable for administration by gene gun, However, other types of microparticles with antigens may be administered in this manner. For example, antigens derived from malaria libraries, DNA or plasmids have been shown to be effectively administered by gene gun in accordance with the procedure described in Smooker PM *et al*, "Expression library immunisation protects mice against a challenge with virulent malaria." Vaccine, 18(22): 2533-2540, 2000, incorporated herein in its entirety by this reference. Vaccination may be by single or multiple dose administration or via prime-boosting.

In a further embodiment the invention provides a method of eliciting an immune response in a subject said method comprising administering to a subject an immunologically effective amount of a composition comprising at least one antigen associated with microparticles, wherein the microparticles are in the same size range as viruses.

The subject may be any human or animal in which it is desired to elicit an immune response. This includes domestic animals, livestock

(such as cattle, sheep, horses, cows, pigs, goats, llamas, poultry, ostriches, emus) and native and exotic animals, wild animals and feral animals.

The term "comprising" has the same meaning as given above.

An immunologically effective amount refers to an amount sufficient to generate an immune response in the subject, preferably after a single administration. This will vary depending on a number of factors including those discussed above, and may depend on whether the subject is a human or animal, its age, weight and so on.

The terms "antigen" and "associated with" have the same meanings as given above.

The term "immune response" refers to the cellular and humoral responses as described above, and also to the response by cells that assist in mounting or amplifying the immune response as described above. In particular the immune response may be provided by the proliferation and/or expansion of dendritic cells, particularly DEC205+, CD40+ and CD86+ cells.

The term microparticle has the same meaning as given above. Preferably the microparticle is between 0.03 and 0.5μm, more preferably between about 0.03 and 0.15μm, still more preferably between 0.03 and 0.1μm. Even more preferably the microparticle is between about 0.03μm and 0.05μm, even more preferably between about 0.03 and 0.04 or between about 0.19 and 0.049μm. Still more preferably the antigen/microparticle composition is particularly adapted to elicit a strong cellular and/or humoral immune response.

In a preferred embodiment the invention provides a method of eliciting an immune response in a subject said method comprising administering to a subject an immunologically effective amount of a composition comprising at least one antigen associated with microparticles, wherein the microparticles are in the same size range as viruses and the immune response comprises the stimulation and/or

proliferation of dendritic cells. Preferably the microparticles are about 40nm to 50nm, most preferably 40 to 49nm in size.

In another embodiment the invention provides a method of eliciting a protective immune response to an antigen via a single dose said method comprising administering, once only to a subject, an immunologically effective amount of a composition comprising at least one antigen associated with microparticles, wherein the microparticles are in the same size range as viruses and the immune response comprises the stimulation and/or proliferation of dendritic cells. Preferably the microparticles are about 40nm to 50nm, most preferably about 40 to 49nm in size.

In another embodiment the invention provides a method of in vivo delivery of an antigen to dendritic cells in order to elicit an immune response said method comprising administering to a subject an immunologically effective amount of a composition comprising at least one antigen associated with microparticles, wherein the microparticles are in the same size range as viruses and the immune response comprises the stimulation and/or proliferation of dendritic cells. Preferably the microparticles are about 40nm to 50nm, most preferably about 40 to 49nm in size.

The invention also extends to a method of producing an immunogenic microparticle/antigen composition comprising contacting microparticles which are in the same size range as viruses with one or more antigens such that the microparticles and antigens become associated. Those skilled in the art will be familiar with the techniques used to produce such a composition.

Detailed description of the invention

The invention will now be described with reference to the following non-limiting examples and figures.

FIGURE 1: Panel A - *Differential uptake of particles of different sizes by macrophages compared to dendritic cells.* 1000 fluorescent

beads of 0.02, 0.1 or 1 micron size per cell were incubated overnight with cultured peritoneal exudate macrophages or bone marrow derived dendritic cells from C57BL/B6 mice and the percentage of fluorescent cells assessed by FACSCan. One of three similar experiments is shown. Similar differences in uptake of different bead sizes were obtained using 10 fold higher bead concentrations, a 3 hour pulse with beads or Balb/c derived antigen presenting cells. Panel B - *Virus sized particles are preferentially found in lymph node cells in vivo.* LEFT C57BL/B6 mice were inoculated intradermally in the footpad with 50μl of 0.1% solution of fluorescent beads of different sizes (0.02, 0.04, 0.1, 0.2, 0.5, 1 and 2 micron) and the draining popliteal lymph nodes removed 10 days later to assess the percentage of cells that have taken up the beads by FACScan. The data is shown as mean percentage of fluorescent cells +/- Standard error (SE) of triplicate samples. The 0.04 and 0.1 micron particle sizes had significantly higher uptake to any other sized particles ($p>0.001$). In similar experiments using comparatively only 0.1 or 1 micron beads, 0.1 micron bead uptake was significantly higher than 1 micron in lymph nodes collected also at days 3, 6 or 9 after inoculation; Panels C & D - *Virus sized particles are taken up preferentially by lymph node NLDC145+ (also known as DEC205+) (panel C) and F4/80+ (panel D) cells.* Lymph node cells that have taken up fluorescent particles were assessed by FACScan analysis for co-expression of the dendritic cell marker NLDC145/DEC205 or the macrophage/monocyte marker F4/80. The data shows the percentage of NLDC145+ or F4/80+ cells that have become fluorescent due to bead uptake.

FIGURE 2: Panel A - *Induction of IFN γ producing CD8 and CD4 T cells by immunization with OVA conjugated to beads of different sizes.* C57BL/B6 mice were immunised intradermally twice (10 days interval) with 100μg of OVA conjugated to 0.02,0.04,0.1,0.2,0.5,1 or 2 micron size beads and spleen T cell activity assessed 10 days after the booster immunisation by IFNγ ELISPOT. Responses were measured to

the H-2Kb restricted CD8 T cell epitope SIINFEKL or to whole OVA. In the case of assessing reactivity to OVA spleen cells were depleted from CD8 T cells before the assay with magnetic beads (Dynabeads) to quantify OVA reactive CD4 T cells. SIINFEKL was used at 2.5 μg/ml and OVA at 25 μg/ml. One of three similar experiments is shown. Two mice per group were immunized for each bead size. ELISPOT cultures were done in duplicates and average values of spot forming units (SFU) per million cells tested are shown. The standard deviation (SD) was always less than 20% of the mean. Panel B - *Correlation between T cells with cytotoxic activity and IFNγ secreting T cells by ELISPOT in response to SIINFEKL* C57BL/B6 mice were immunised with beads-OVA of different sizes and reactivity to SIINFEKL assessed by IFNγ ELISPOT as described above. In addition, the number of SIINFEKL specific T cells with cytotoxic activity was determined in parallel by limiting dilution analysis. Chromium loaded EL4 cells alone or pre-pulsed with 2.5 μg/ml of SIINFEKL were used as targets. The data shown illustrates the strong correlation (R square= 0.9254) found between the two assays. One of two similar experiments is shown. PANEL C - *Antibody production induced by immunisation with OVA conjugated to beads of different sizes.* Serum was collected from the mice described in Panel A and serum dilutions tested for OVA specific IgG reactivity by ELISA. Individual mice receiving 0.02, 0.04, 0.1, 0.2, 0.5, 1 or 2 micron size OVA-bead immunisation are plotted. One of two similar experiments is shown.

FIGURE 3: *Covalent conjugation of antigen to beads necessary to induce optimal T cell responses.* Panel A - Bead-conjugated OVA alone accounts for MHC class I restricted T cell responses C57BL/B6 mice were immunized with OVA conjugated covalently to 0.04 micron beads without prior dialysis (Control) or following dialysis against PBS through a 300Kd exclusion membrane (Dialysed). The induction of IFNγ producing splenic SIINFEKL specific CD8 T cells was assessed 10 days after one intradermal immunization by ELISPOT. The mean +/-

SE for 4 mice per group assessed by ELISPOT in duplicate wells is shown. PANEL B - Co-administration of beads and soluble OVA is not sufficient to induce optimal MHC class I restricted T cells responses. C57BL/B6 mice were immunized with OVA conjugated covalently to 0.1 micron beads (Beads conjugated-OVA) or mixed with OVA prior to injection (Beads/OVA mixed). The induction of IFNγ producing splenic SIINFEKL specific CD8 T cells was assessed 10 days after one intradermal immunization by ELISPOT. The mean T cell precursor frequency for 2 mice per group assessed by ELISPOT in duplicate wells is shown.

FIGURE 4: *A single immunization with viral sized beads-OVA is sufficient to induce long lasting high levels of MHC class I restricted T cells.* Panel A - C57BL/B6 mice were immunized intradermally once, two or three times with beads-OVA (0.1 micron), each time 14 days apart and their IFNγ response to SIINFEKL examined in each case 10 days after the last immunization by ELISPOT. Three mice were immunised per group and the data shows the mean of duplicate assays on each mouse. One of two similar experiments is shown. Panel B - Mice were immunized once with beads- OVA (0.1 micron) and IFNγ responses to SIINFEKL or OVA tested by ELISPOT 12 or 82 days later. Antibody levels to OVA measured as in Figure 2 were maintained at day 82.

Figure 5: Assessment of CD40 expression by cells that have taken up beads in the draining lymph node after intra-dermal immunization Popliteal LN cells from naive C57BL/6 mice (left) or mice immunized with fluorescent 0.04μm fluo-beads intradermally in the footpad (right) were dissected 48 hours after injection and analysed for expression of CD40 by staining with PE conjugated antibodies specific to these markers. FL-1 = FITC positive cells (bead+) and FL-2= PE positive cells (marker+). Background staining was negligible (<1%). The left panel represents popliteal LN cells from non-immunised

animals, the right panel represents the same type of cells from VSP-OVA immunised animals.

Figure 6: Induction of immature and mature murine DC proliferation in response to 0.1μm OVA-beads. DCs were cultured from bone marrow cells extracted from the tibia and femur of the hind legs of C57BL/6 mice, with the addition of GM-CSF and IL-4. After 5 days in culture, the cells were separated into the experimental conditions at 1.25 x 10^6cells/1.25ml and pulsed with conjugated beads to OVA (0.1μm) at 1000 beads/cell. After 4 hours of pulsing, LPS and TNFa were added to appropriate cultures. The cells continued to incubate overnight, and proliferation thymidine assay set up the next day and incubated overnight. Each value represents triplicate averages ± SD (*p<0.00001 between unpulsed DCs and experimental groups, unpaired t-test).

Figure 7: Phenotypic characterisation of APC taking up 0.04 compared to 1μm particles *in vivo* C57/B6 mice were injected in the footpad with 50μl of 0.04 or 1μm fluobeads-OVA. Draining popliteal LN were analysed 48 hours later for co-staining of bead positive cells with cell markers of activation and antigen presenting cell lineage, the mean +/- SE for 3-14 mice/marker is shown. 0.04 and 1μm fluobead+ cells had significantly different expression of DEC205, F4/80, CD40, CD80 and CD86 (p<0.05).

FIGURE 8A: Mechanism of viral size particle uptake by DC. Bone marrow derived cultured DC were incubated with phrobol myristate acetate (PMA) at 0 (black),5 (white),10 μM (grey); amiloride (AML) at 0 (black),1 (white), 3 mM (grey) (white), or cytochalasin D (CDD) at 0 (black), 0.25 (white) or 0.5 μg/ml (grey) for 30 min and 0.04 μm-0VA-fluorescent particles added a further 3 hours. Selective inhibition of caveolae, clathrin coated pit formation, or phagocytosis has been reported for 10 μM PMA, 3 mM amiloride and 0.5 μg/ml CCD, respectively 14, 15. When used together PMA was kept constant at 10 μM and AML was added at 1 (white) or 3 mM (grey). The number of fluorescent cells was

assessed by FACScan. Data is presented as the mean +/- SD of triplicate cultures.

FIGURE 8B: Confirmation of the mechanism of uptake DC were incubated with nothing, CDD 1 μg/ml, filipin (FIL) at 1 μg/ml or ammonium chloride (AC) at 40 mM for 30 min and 0.04 μm or 1 μm fluorescent beads added a further 3 hours. Selective inhibition of caveolae or clathrin coated pit formation has been reported for 1 μg/ml filipin and 40 mM ammonium chloride, respectively 14-17, 29. The number of fluorescent cells was assessed by FACScan. Data is presented as the mean +/- SD of triplicate cultures.

Figure 9: Soluble OVA and 1 um-OVA beads fail to induce comparable protection to 0.05 um-OVA beads. C57/B6 mice were immunised ID with OVA conjugated to 0.05 um or 1 um beads, soluble OVA or left untreated and then challenged as above. Data is presented as the individual tumour sizes at day 10 for 8 animals in each group. One of two similar experiments is shown. The difference in the frequency of tumours between the 0.05 um-OVA bead group and each one of the other groups was significant: P=0.0001 vs. naïve; p=0.0007 vs. soluble and p=0.0035 vs. 1 um bead-OVA.

FIGURE 10: Viral sized particles do not co-localize with early endosomes (LEFT). Bone marrow derived DC were incubated overnight with 0.1 micron beads-OVA (500 beads/cell), washed gently to remove free beads and prepared for confocal microscopy by spinning onto glass slides. Cells were then fixed in paraformaldehyde, permeabilised with triton and stained the presence of the early endosomal marker Rab4 using a biotin conjugated monoclonal antibody followed by streptavidin-Alexa. Similar results were observed with unconjugated 0.04 and 0.1 micron beads and one of three experiments is shown. Fluorescent 0.1 micron beads similarly failed to co-localise with Rab4 staining using DC incubated with beads for 30 minutes or for 3 hours. (RIGHT) Mice were injected intradermally in the hind footpad with 0.1 micron beads-OVA and the draining popliteal lymph nodes

dissected 48 hours later for confocal analysis as described above. No co-localization was observed for the Rab4 marker and OVA conjugated or unconjugated 0.1 micron or 0.04 micron beads. One of three experiments is shown. By contrast co-localization was confirmed for the positive control mice immunized with 1 micron sized fluorescent beads.

FIGURE 11: Protection against tumour PANEL A C57/B6 mice immunised intradermally (ID) once with OVA-VSSP (immunised) or left untreated (naïve) were challenged 30 days later subcutaneously with $5x10^6$ EG7 (tumour cells) . Tumours were measured using calipers. Individual tumour growth curves for 10 animals per group are shown. PANEL B Tumours were induced as above and at day 8 of tumour growth (day 0 of immunisation) 6 animals left untreated (Naïve) and 6 immunised ID with OVA-VSSP (Immunised). Individual growth curves are shown day 3-13 after immunisation.

FIGURE 12: Survival of mice to lethal malaria challenge after VSSP immunisation. C57/B6 mice immunised intradermally once with 100 μg of VSSP-OVA, VSSP-lysate or lysate alone were challenged with 500,000 lethal Plasmodium yoelii 17XL infected C57/B6 red-blood cells. Survival was monitored daily. 5 animals were challenged per group and one of six representative experiments is shown. In similar experiments naïve mice had 40% survival after 2 weeks (8/20 mice). The lysate was generated by repeated freeze-thaw of P. yoelii 17XL infected red-cells and ultra centrifugation and conjugated to VSP using the standard protocol.

FIGURE 13: The antigen nm23 was conjugated to 0.05 μ bead (VSP) as described before for the antigen OVA, injected intradermally into mice at 100μg/mouse and 10 days later IFN gamma reactivity assessed in the spleens of immunised animals by ELISPOT. The data id presented as the precursor frequency of cells responding to nm23 per million spleen cells as spot forming units (SFU/million) ± the standard devation of the mean (SD). The individual responses of three mice (m1-m3) are shown.

FIGURE 14: The cancer antigen nm23 or OVA were conjugated to VSP per standard protocol and 100 μg/mouse injected intradermally. 10 days later the induction of IL4 secreting cells was assessed by ELISPOT. Data is presented as SFU/million +/- SD for three individual mice immunised with each immunogen.

FIGURE 15A: Antibody reactivity to OVA in the sera of mice immunised once intradermally with 0.05μm beads conjugated to OVA (VSP-OVA) and assessed 90 days later by ELISA (B group) in comparison to non-immunised controls (A group).

FIGURE 15B: The same sera from mice in Figure 15A was tested for the presence of OVA specific IgE antibodies by ELISA, in two naïve mice (A2 and A3) and three VSP-OVA mice (B2, 3 and 5).

FIGURE 16: PANEL A Induction of long lasting antibody responses by a single immunisation. C57/B6 mice were immunised once with OVA conjugated to 0.04μm beads and sera collected at different time-points. The mean optical density at 405nm +/- SE for each group of four animals in OVA specific IgG ELISA is shown. Naïve sera is shown as negative control. One of two similar experiments is shown. Similar ELISA results were obtained for total Ig and no IgM or IgA was detected (not shown). OVA alone failed to induce IgG responses over PBS immunised animals and OVA in Complete Freunds Adjuvant (CFA) induced IgG responses a log higher than single dose 0.05μm beads-OVA (not shown). PANEL B Induction of long lasting high levels of IFNγ producing T-cells by a single immunisation with 0.04 μm beads OVA C57/B6 mice were immunised ID once with OVA conjugated to 0.04μm beads (black or chequered bar), soluble OVA in PBS (white bar) or with OVA mixed in with 0.04μm beads (grey bar). Precursor frequency of SIINFEKL reactive spleen T-cells was assessed 10 days later (back, white and grey bars) or 12 months later (chequered bar) by IFNγ ELISPOT. Four mice were tested per group and one of two similar experiments is shown. Average values of spot forming units (SFU) per million cells +/- standard deviation (SE) are shown for each

group. In similar experiments using 10 times less antigen (10 μg VSP-OVA) a single immunisation induced 102 +/- 56 SIINFEKL specific spleen cells per million (n=4). Cytotoxic T-cell responses in standard Chromium release assays were also observed 10 days after a single immunisation (>50% Specific lysis for 3/3 animals at E:T ratio 20:1; not shown).

Figure 17: Prime/boost C57/B6 animals were left untreated (Nothing) or primed intradermally with 100ug of peptide cp13-32 from MUC1 conjugated to 700ug of KLH in Complete Freunds Adjuvant (cp13), mannan conjugated recombinant MUC1-GST fusion protein (MFP) or 0.1um VSP conjugated to MUC1-GST fusion protein (VSP). 14 days later animals were boosted intradermally with a million infectious vaccinia virus expressing the MUC1 protein, and reactivity to the epitopes in cp13-32 assessed by IFNg ELISPOT 10 days later. The data shown is the mean number of IFNg producing cells +/- SE per million spleen cells averaged for 2-3 animals/group.

Figure 18: Comparison of polystyrene and glass 0.05 um VSP-OVA particles. Polystyrene 0.05μm beads were conjugated to OVA as before (PS) and compared to OVA conjugated glass beads in the same way (G1). In addition, a different chemical procedure was compared for the glass beads. Briefly, glass beads were weighed and suspended to 2.5%solids in PBS and washed twice. PBS was removed by 5 minute centrifugation in a microfuge. The bead pellet was resuspended in 8% gluteraldehyde in PBS ph 7.4 and mixed gently at room temperature overnight. The beads were then washed 3x with PBS resuspended in PBS and 500μg of protein per ml was added and mixed gently for 5 hours. The beads were then pelleted and the reaction was stopped by resuspending the pellet in o.5 M ethanolamine and mixing for 30 minutes. The beads were then washed in PBS and used for immunization (G2). Polystyrene (PS) or glass (G1 or G2) VSP-OVA were immunised intradermally at 100 ug/mouise and SIINFEKL specific IFNg secerting T cells quantified 10 days later from spleens by

ELISPOT. The data shows individual mean+/- SE for three animals per group.

Figure 19: Mode of bead conjugation and immunogenicity. Ovalbumin at 2 mg/ml in 50 mM MES buffer (ph 6.0) was mixed with the polystyrene carboxy modified 0.05 μm beads (2% solids) for 15 minutes. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiiamide was added to each preparation at 4 mg/ml (pH 6.5) and incubated at room temperature for 2 hours. The standard (Glycine) was quenched with 7 mg/ml of glycine or 20 μl of l M ethanolamine (amine) pH 7.4, or 20 μl of 1 M aminoacetaldehyde dimethyl acetal (aldehyde) pH 8.0, or 20 μl of 1 M ethylenediammne (alcohol) pH 7.4. The preparations were incubated at room temperature for approximately 16 hours. All the preparations were dialysed overnight in PBS at 4°C. The aldehyde preparation was quenched further with 20 μl of 1 M HCL and incubated for 4 hours, and dialysed overnight in PBS at 4°C. 2-3 C57/B6 mice were immunised with 100 ug intradermally of each one of these VSSP-OVA particles and immunogenicity assessed in spleens by IFNg ELISPOT to the CD8 T cell epitope SIINFEKL. Results are shown as mean+/-SE of SFU/million spleen cells for each animal.

Example 1: Materials and Methods used

Mice and Immunizations C57BL/6 and BALB/c 6- to 8- week-old mice were purchased from the Walter and Eliza Hall. Mice were immunized with 100μl of antigen conjugated beads intradermally (ID) in the hind footpads.

Reagents: All reagents including the antigen Ovalbumin (OVA, Grade III) and 1-Ethyl-3-(3-DimethylAminopropyl)Carbodiamide (EDAC) were purchased from Sigma unless otherwise stated. Monoclonal antibodies for FACScan and confocal studies were either purified in house from hybridoma lines on a Protein G column (Pharmacia) or purchased from Pharmigen. FITC conjugated and carboxylated fluospheres 0.02-2μ were purchased from Molecular Probes and non-fluorescent

carboxylated microspheres from Polysciences. Abs to the following markers were used: MHC II, MHC I, CD11c, CD11b, F4/80, NLD-145, CD8alpha, CD40, CD80 and CD86. The anti-Rab4 monoclonal antibody used in confocal studies was the kind gift of Dr. Russel (Peter McCallum Research Institute).

Antigen presenting cells: Denditric cells were prepared from bone marrow monocytes with minor modifications of previously published methods [5]. Briefly cells were harvested from tibia and long bones of the hind limbs by flushing out the cells from the bone cavities with media, following by red cells lysis. The cells were plated out at $1x10^6$ cells/ml in RPMI ((CSL, AUST) supplemented with 10% heat inactivated foetal calf serum (FCS), 4mM L-glutamine, 100 U/ml penicillin, 100mg/ml streptomycin sulphate and 100 μM β-mercaptoethanol. and GM-CSF at 1000 units/ml and IL-4 at 0.2 ng/ ml were added. The 10 ml cultures were grown for 5-6 days in petri-dishes of a 100mm diameter at 37C in a humid CO_2 incubator. Macrophages were recovered from the intraperitoneal (IP) cavity of mice three days after IP injection of thioglycollate, and cultured for 3 days to enrich for adherent cell fractions as described [6].

Bead-antigen conjugation was performed following the manufacturers instructions. Briefly, OVA was diluted to 2.0 mg/ml in 0.05M MES buffer pH 6.0 mixed in a volume ratio of 1:1 with beads of 2% solids/volume. The mixture was rocked gently for 15 minutes and then 4 mg/ml EDAC was added. The pH of the mixture was adjusted to 6.5 with dilute NaOH and the mixture was rocked gently for two to three hours. The reaction was stopped with glycine to a final concentration of 100 mM. After 30 minutes of mixing the preparation was dialysed overnight in the cold in PBS. The preparation was either used immediately or stored at 4^0 C with 0.01% azide for later use.

Cytotoxicity assays: These were performed as described [3]. Briefly, effector cells for cytotoxicity assays were generated by culturing spleen cells for 7 days at 2.5×10^6/ml in 2 ml well plates at 37°C in a humid CO_2

incubator with 10μg/ml of the peptide antigen in RPMI medium (CSL, AUST) supplemented with 10% heat inactivated foetal calf serum (FCS), 4mM L-glutamine, 100 U/ml penicillin, 100mg/ml streptomycin sulphate and 100 μM βmercaptoethanol. Interleukin 2 (10 U/ml, recombinant human IL2, Lymphocult HT, Biotest, UK) was added on day 3. Targets were ^{51}Cr loaded EL4 cells, alone (background) or pre-pulsed for 1h at 37 °C with 10μg/ml of the SIINFEKL peptide or EG7 an ovalbumin transformed EL4 cell line. Unless otherwise stated assays were performed in duplicate at an effector:target ratio of 20:1. Spontaneous lysis (with media alone) and maximum lysis (with 5% triton) were set up for all targets in quadruplicate. Supernatants were harvested after 4h. %Lysis was calculated as 100x ((Experimental release -Spontaneous release)/ (Maximum release-Spontaneous release). % Specific Lysis (%SL) was %Lysis with peptide- %Lysis with no peptide.

Cytotoxic T cell precursor(CTLp) assays: CTLp assays were performed as described previously [7] CTLp frequencies were determined from a minimum of 32 replicates, for at least 6 effector cell numbers (1x103 - 1.28x105). Cells were cultured in U-bottomed microtitre trays, with 5x105 mitomycin C treated syngeneic spleen cells, in DMEM supplemented with 10% foetal calf serum, 5μM of SIINFEKL or OVA and 10 U/ml rhIL-2. Seven days later, each microculture was assayed for cytotoxicity by replacing 100 ml of culture medium with 100 μl target cell suspension containing 104 51Cr-labelled EL4 OR EG7 as targets. Cytotoxic activity was considered to be present if in each well 51Cr release was found three standard deviations above the mean isotope release from 104 effectors cultured with stimulators only or from stimulator cells with peptide only or rIL2 only. A linear relationship ($0.987 \leq r2 \leq 1$) existed between the number of responder cells, represented on a linear scale, and the frequency of negative wells on a logarithmic scale. CTLp frequencies were determined as the inverse of responder cell dose required to generate 37% negative wells[8, 9].

CTLp frequency assays were performed three times and the individual frequencies did not differ by more than 20% from the mean value.

ELISPOT IFNγ assays: These were performed as described [3]. Briefly, 100 μl of 5 x 10^6 /ml freshly isolated spleen cells were incubated with the stated stimuli for 18 hours on mixed acetate plates (MAHA Millipore) pre-coated with an anti-murine IFNγ mAb (R4), (EACC). Duplicate wells were set up for each condition. The media used was RPMI 1640 (CSL) supplemented as described above. After overnight incubation cells were washed off and the plates incubated with a second biotin conjugated mAb to murine IFNγ (XMG.21-biotin,Pharmigen, CA, USA), followed by an extravidin alkaline phosphatase (A-AP) conjugate at 1 μg/ml (Sigma). Spots of alkaline phosphatase activity were detected using a colorimetric AP detection kit (Biorad, Hercules, CA, USA) and counted utilising a dissection microscope. The data are presented as spot forming units (sfu) per million cells. The SIINFEKL peptide was utilised at 2.5 μg/ ml.

Statistical analysis in protection studies the number of mice protected in each group was compared using a χ^2 test in the Statcalc program in the EpiInfo Version 5.0 package. In immunogenicity studies the ELISPOT and Chromium release responses were compared between groups using the Student's t test with the Microsof Excel Version 5.0a package. Linear regression analysis was used to assess correlation between immunogenicity and protection using the SPSS for Windows statistical program package.

Bead uptake by dendritic cells and macrophages Three day old macrophage cultures grown on microscope slides and five day old dendritic cell cultures were fed with different size fluorescent microbeads for periods of 5 minutes to 24 hours. The cultures were then washed to remove free beads and prepared for FACScan or confocal analysis.

Analysis of cells taking up beads in vivo: Draining lymph nodes and spleens were collected from bead immuninuzed mice at various time intervals from 12 hrs post immunization and up to 12 days. Cells were

collected and after red blood cell lysis and washing they were prepared for FACscan or confocal analysis.

Cell surface marker staining and flow cytometry: For surface staining, $5x10^5$ cells were incubated with PE-labelled MoAb to surface markers F4/80 and NLD-145, CD. In cases where the antibody was not directly labelled after two washing steps the cells were incubated with a second PE-labelled antibody specific for the first antibody. Naive serum of the species where the second antibody was raised was used in a blocking step before incubation with the second antibody. After two washes, the cells were washed with PBS/0.2% paraformaldehyde and analysed with a FACScan flow cytometer (Becton-Dickinson) and CellQuest software. Light scatter gates were set to exclude dead cells and nonlymphoid cells. Cells from bead immunized unstained for any surface marker and cells from naive mice stained with a PE-labelled antibody to a surface marker were used to determine compensation for overlap between the FITC and PE emission spectra.

Confocal microscopy of phagocytosed FITC-labelled beads: An Olympus scanning confocal microscope was used with a Krypton-Argon laser source equipped with dual fluorescence and transmission detection to determine whether fluoresceinated beads of different sizes were phagocytosed by macrophages or dendritic cells. Serial sections through the samples were acquired at step sizes between 0.5-1.0 microns to determine phagocytosis and analysed on Optiscan Analyzer. Cells were excited at 488nm and 568nm for fluorescein and Alexa 594 respectively and detected through 530nm and 610nm band pass filters respectively. Throughout acquisition, laser power was kept below saturation levels and gain and offset parameters maintained within individual experiments.

ELISA assays: Antibody responses to OVA were measured using ELISA. Polyvinyl chloride microtitre plates were coated with OVA (10 μg/ ml in 0.2 M NaHCO3 buffer, pH 9,6) overnight at 4°C. The plates were washed 4x with PBS/0.05% Tween20and 4x with PBS and then

blocked for non-specific binding with 2% bovine serum albumin for 1 h at room temperature. After washing as above serial dilutions of the mouse sera were added and incubated for a further 1 h at room temperature. Non immune mouse serum was used as the negative control. The plates were washed and the bound antibody detected using horseradish-peroxidase-conjugated sheep anti-mouse Ig (Selinus, AUS) and the chromogenic substrate 2,2"-azino-di(3-ethylbenzthiazoline) sulphonate (Amersham, UK). The absorption at 405 nm was recorded using an EL 312e microplate reader.

Example 2: *Preferential uptake of VSSP by antigen presenting cells in vitro and in vivo*

A number of studies using cells from the macrophage/monocyte lineage have shown particle size dependent phagocytosis, with optimal uptake at a 1 micron diameter [10, 11]. Uptake has been observed in dendritic cells, however, a comprehensive range of particle sizes has not been tested. The inventor was specifically interested to establish whether protein coated particles of viral size (0.03-0.1μm), would be efficiently taken up by dendritic cells or macrophages. Figure 1a shows that thioglycollate elicited peritoneal exudate macrophages internalised both 1μm and 0.1μm fluorescein-labelled particles (fluo-beads). Immature bone marrow derived dendritic cells, by contrast, were found to take in preferentially 0.1μm sized fluo-beads. Confocal microscopy was used to confirm the particles were inside of the cells (not shown). This *in vitro* data suggested viral sized solid particles (VSSP) could also be preferentially taken up by antigen presenting cells *in vivo*. Fluorescent polysterene protein conjugated particles in a range of sizes (0.02, 0.04, 0.1, 0.2, 0.5, 1 and 2μm) were injected intradermally (ID) into the footpad of C57BL/B6 mice and cells from the draining popliteal lymph nodes collected 10 days later for FACScan analysis. Particles of the 0.04-0.1μm size were taken up preferentially by lymph node cells (Figure 1b). Similar results were obtained analysing lymph node cells

on days 1, 3, 6 and 10 after particle injection, and with unconjugated particles. VSSPs were efficiently taken up by antigen presenting cell expressing both macrophage and dendritic cell surface markers (Figure 1b). Bone marrow derived dendritic cells *in vitro* also took up VSSPs. As expected, these cells were of a predominantly myeloid phenotype [12].

Example 3: *VSSP prime high precursor frequencies of cytotoxic and IFNγ secreting T cells as well as antibodies.*

Efficient VSSP uptake by dendritic cells in vivo suggested their use for targeted antigen delivery and potential as novel vaccines. C57/BL mice were immunised with ovalbumin (OVA) coated particles of 0.02, 0.04,0.1,0.2,0.5,1 or 2μm, boosted after 15 days and serum or spleen cells collected 10 days later. Figure 2a shows optimal induction of IFNγ secreting CD8 T cell induction to the MHC class I restricted SIINFEKL epitope achieved using 0.04μm sized particles. CD4 T cells responding to OVA were found at similar precursor frequencies with OVA conjugated particles ranging from 0.04-1 micron. Cytotoxic T cells to SIINFEKL were also induced with VSSPs and correlated with the precursor frequency of IFNγ secreting T cells (R2=0.92)(Figure 2b). Surprisingly, OVA-specific IgG was also found at the highest precursor frequencies in mice immunised with 0.04μm particles, followed by those immunised with 1μm particles (Figure 2c). Similar immunogenicity results were found with using VSSPs without fluorescein. Thus, in contrast to many immunogens and adjuvants promoting preferentially a cellular or a humoral response, VSSPs were capable of inducing high levels of both.

The results shown in Figure 2 are based on injecting the same total amount of antigen after conjugation without eliminating residual soluble antigen. Figure 3a shows that similar T cell responses were obtained with 0.04 micron VSSPs after the soluble antigen was eliminated by dialysis or ultracentrifugation. Therefore, there was no

significant contribution from the soluble antigen to the observed T cell responses. This was further supported by the comparison of conjugated and unconjugated OVA and VSSP mixes. Figure 3b shows that only covalently conjugated VSSP induced high levels of SIINFEKL specific T cells. Thus, covalent attachment to the VSSPs was necessary to target OVA into the class I presentation pathway and induce class I restricted T cells *in vivo*. It could be argued that a higher amount of conjugated protein in smaller compared to larger particles could by itself result in higher VSSP immunogenicity. However, this was not so, since: 1) Immunogenicity peaked at 0.04μm and 0.02μm sized beads induced little reactivity (Figure 2), 2) 0.04-0.1 micron VSSPs were consistently more immunogenic than 1μm particles independently of the level of antigen conjugation, (3) Increasing the immunising concentration of 1μm particles up to 100 fold (up to 1 mg/mouse) over that used for VSSPs failed to enhance immunogenicity to the levels seen with a range concentrations of antigen conjugated to VSSPs, 4) Immunising with equivalent amounts of bound protein on beads of different sizes, or with the same number of different sized beads, consistently showed VSSPs to be more immunogenic than larger particles across a range of concentrations (0.5-1000 ug total OVA, 0.5-50 ug conjugated OVA and $10^3 - 10^8$ beads per animal).

Example 4: A novel pathway for the uptake and processing of particles

It has been suggested that peptides derived from digestion of exogenous antigen through the class II MHC processing pathway may be regurgitated and subsequently bind to empty class I MHC molecules at the cell surface [1, 10] . This alternative mechanism of class I presentation is independent of transport into the endoplasmic reticulum (ER) mediated by TAP (transporter associated with antigen processing). Hepatitis B surface protein VLPs are processed for class I presentation in macrophages by a TAP independent mechanism [2] . The inventor

immunised TAP knockout C57/BL mice with OVA-conjugated VSSPs. No T cell responses above background levels could be detected to SIINFEKL or OVA in TAP-KO animals suggesting VSSP processing for class I presentation was by contrast, TAP dependent. A TAP dependent mechanism of class I presentation of exogenous antigen has been described for proteins adsorbed onto 1μm particles based on 'leakiness' of endocytic vesicles and accidental release of antigen into the cytoplasm [1, 10] . Processing of such large particles taken up by phagocytosis involves an early conjugation step with lysosomes expressing the Rab4 adaptor protein [13]. The inventor used confocal microscopy to determine whether VSSPs would be routed via this pathway. Figure 4 shows that Rab4 and VSSP fluo-bead containing vesicles did not co-localise either in bone marrow derived dendritic cells in culture, or *in vivo* in lymph node cells 24 hours after intradermal VSSP administration. VSSPs therefore may use a processing pathway which differs from that used by both VLPs or larger particles in that it is Rab4 independent and TAP dependent. The mechanism was further investigated in Example 7.

Example 5: VSSP induce expansion of antigen presenting cells in vivo and in vitro

C57BL/6 mice were left untreated (naïve) or immunized with fluorescent 0.1μm fluo-beads intradermally in the foot pad. Popliteal lymph node (LN) were dissected 48 hours after injection and analysed for expression of CD40 by staining with PE conjugated antibodies specific for this marker.

The results (Figure 5) show that 0.04, 0.05 and 0.1μm polystyrene beads alone or conjugated to OVA were able to increase up to 4 fold the total number of cells recovered from the draining popliteal lymph node after intradermal immunisation. Moreover, they caused 1.5 fold increase in the proportion of NLDC145+ (dendritic cell marker) but not F4/80+ (monocyte/macrophage marker) cells. They also enhanced

>1.5 fold the proportion of lymph node cells expressing the activation molecules CD40 and CD86. Figure 5 shows an example of the increase in CD40+ cells after immunisation observed by FACScan (33% to 63%). It also shows that many of these cells have taken up the 0.04μm beads, in this case we used 0.04μm beads with a fluorescent green core.

0.04, 0.05μm and 0.1 polystyrene beads alone or conjugated to OVA were able to induce dendritic cells purified from mouse bone marrow to proliferation in vitro. Immature, but not mature (after activation with LPS and TFN-alpha) dendritic cells were susceptible to this activating effect of VSSP (Figure 6).

These data together suggest that particles of 0.04-0.1μm in size (VSSP) have the unsuspected and previously unknown ability to stimulate antigen presenting cells, including dendritic cells, and specifically cells expressing potent co-stimulatory molecules like CD40 and CD86 to proliferate and expand. This could further explain why they are so potent. Moreover, it suggests a mechanism by which VSSP may have an adjuvant effect (see Example 14 below) even for responses to antigen when it is not chemically conjugated to them.

Example 6: Extended phenotype of cells taking up VSSP rapidly after injection

To assess further which cells take up VSSP rapidly after intradermal footpad injection (and thus may be responsible for subsequent activation of immunity), the draining popliteal draining lymph node was dissected. The phenotype of cells that had taken up 0.04μm VSSP-OVA with a fluorescent core was then analysed by FACScan and compared to identical particles but which were 1μm in size. Figure 7 shows the proportion of 0.04μm bead+ or 1μm bead+ cells expressing each phenotypic marker. Cells taking up 0.04μm beads were mostly NLDC145+, CD40+ and CD86+. In addition more CD11c+, CD4+ and CD8+ cells were 0.04 than 1μm bead+. This highly

activated DC phenotype of cells that have taken up 0.04μm beads may further explain why the immune responses we observed are so potent, particularly CD8 T cell responses.

Example 7: Uptake and processing of microparticles.

To address the mechanism of 0.04μm bead-OVA uptake by dendritic cells (DC), bone-marrow derived DC were incubated with inhibitors of phagocytosis (cytochalasin D, CDD); clathrin pit (amiloride, AML) or caveole mediated internalisation (phorbol myristate acetate, PMA) [14, 15]. DC were cultured in triplicate with PMA, AML, CDD, filipin (FIL) or ammonium chloride (AM) (all from Sigma) at the stated concentrations for 30 min. OVA-fluorescent 0.04 μm beads were added for a further 3 hours and uptake was quantified by FACScan.

PMA and AML both decreased 0.04 μm beads-OVA uptake by DC, whereas CCD failed to cause any inhibition (Fig. 8a). Inhibition by PMA and AML was additive (Fig 8a). This suggested clathrin pits and caveole could both be involved in VSSP uptake.

Amiloride acts by inhibiting Na+-H+ exchange necessary for receptor mediated endocytosis [14]. The inventor tested additionally ammonium chloride which inhibits the assembly of clathrin pits by interfering with cytosol acidification [14]. Ammonium chloride inhibited uptake of 0.04 but not of 1μm size beads (Fig. 8b), confirming a role for clathrin pits in VSSP uptake. Caveole have been suggested to mediate a novel mechanism for uptake of viral particles in DC [15]. The inventor results using PMA suggest that caveole were involved in VSSP uptake in DC (Fig. 8a). To confirm this, the inventor used another inhibitor of caveole, filipin. Filipin acts through cholesterol sequestration, whereas PMA affects the phosphorylation events regulating caveole internalisation [14, 15, 16, 17]. Similarly to PMA, filipin blocked 0.04μm but not 1μm bead uptake, confirming the inventor's hypothesis (Fig 8b).

Caveole and clathrin pits can convey molecules to endosomal and lysosomal compartments [14, 16]. Alternatively, caveole may

deliver antigen directly into the cytosol [17] leading to cytoplasmic processing and TAP dependent transport into the endoplasmic reticulum for presentation with MHC class I [1, 18].

These results, together with those shown in Example 4, indicate a novel pathway for processing of antigens presented in accordance with the present invention. The immunogenic composition of the invention appears to be taken up by antigen presenting cells via caveole and/or clathrin pits, after which the antigens are processed by Rab4 independent and TAP dependent pathways for MHC class I presentation. This observation is novel in that the ability of caveole to induce the TAP-dependent antigen processing pathway and CD8 cells has not been reported before.

Example 8: Comparison of protective efficacy

50 nm (i.e. 0.05μm) (VSSP) OVA conjugated particles were compared directly with OVA alone or 1000nm (i.e. 1μm) OVA conjugated particles, for ability to protect mice against subsequent subcutaneous challenge with 100,000 tumour cells (EL4) expressing OVA. All mice were immunised with 100 μg of either of the above (or nothing=naïve) intra-dermally once and then challenged with tumour 30 days later. Figure 9a shows that VSSP-OVA prevented completely the growth of OVA expressing tumours, whereas OVA alone or with 1000 nm beads had a non-significant effect on protection.

Example 9: A single VSSP immunization induces high levels of immunity and protects against tumor challenge.

Intradermal immunisation with OVA-conjugated VSSPs induced IFNγ producing and cytotoxic SIINFEKL specific T cells (Figure 10a and 1a and b). Additional immunizations did not further increase reactivity (Figure 10b and 10c). T cells could be maintained at high precursor frequencies 82 days after immunization (Figure 10d). Antibody responses were similarly maintained (Figure 16). The CD8 T cell

precursor frequency levels achieved by a single VSSP immunization were higher than those observed for single, or even multiple doses of VLP particles and are only comparable to the highly efficient heterologous prime/boost regimes [1, 2, 3, 19, 20]. High IFNγ producing and cytotoxic T cell precursor frequencies are associated with protection against many intracellular pathogens and cancer [21, 22, 23]. The inventor immunised C57/BL mice with a single intradermal dose of OVA-conjugated VSSPs and then challenged them with the EG7 tumor cell line, which expresses cytoplasmic OVA and is a target for cytotoxic SIINFEKL specific T cells *in vitro*. The results show that VSSP immunised mice were completely protected against tumor challenge, whereas all the naïve controls developed tumors. In addition, antibody levels were also increased following a single administration of antigen conjugated VSSP, similarly to those observed in Figure 2c.

Further work on VSSP vaccines

Examples 10 to 13 described below formally demonstrate that VSSP can be used with a variety of antigens and induce broad immunity comprising both IFN and IL4 producing T cells. High levels of IgG, but not the potentially allergenic IgE antibodies are also induced after a single dose.

The effectiveness of VSSP for therapy is shown in an additional two models. 1) 100% clearance of established tumours (see Example 10) and 2) protection against lethal malaria after a single administration of the vaccine (see Example 11). This further confirms VSSP as an unusually potent and flexible vaccination protocol to develop single dose vaccines against a variety of diseases. Moreover, on the basis of these findings the inventor believes that VSSP may be used for therapy as well as prevention of cancer.

Example 10: Clearance of established Tumours

The inventor has observed that a single immunisation with beads-OVA protects completely against subsequent challenge with tumours expressing OVA.

C57/B6 mice were immunised once with 100μg bead(0.05μm)-OVA (ID) or left untreated (naïve). After 30 days, mice were challenged with $5x10^6$ EG7 tumour cell lines. Tumour size was measured using calipers on days 3-13 after immunisation. For regression studies, mice were given the EG7 cells and eight days later divided into groups of similar tumour size distribution. One group was left untreated and the other was immunised with bead-OVA after 3 days (ie day 11 after administration of tumour cell line).

The inventor now shows that already established tumours can be cured by a single immunisation into a tumour bearing mouse. Tumours were cleared from immunised mice within two weeks after a single injection. This therapeutic ability is highly unusual for any cancer vaccine, and makes this vaccination vehicle highly promising for development of a therapeutic vaccine (Figure 11B).

Mucin-1 or Muc1 is a breast cancer associated antigen. Immunisation once with VSSP-Muc1 protein also inhibited tumour formation in mice challenged with tumour cell lines expressing the breast cancer antigen (see Figure 11A).

Example 11: Protection Against Malaria

The inventor demonstrates that polysterene beads of 0.05 μm in diameter may be used as a vehicle to induce protection against malaria in mice. A lysate from *Plasmodium yoelii* infected red cells was conjugated to beads and used to immunise mice which were then challenged with a lethal dose of the parasites.

Blood was collected from C57BL/6 mice infected with *P. yoelii 17XL* at 50% parasitaemia. Red blood cells (RBC) recovered after

centrifugation 800g for 15 min were freeze/thawed three times and sonicated (lysate). Lysate was conjugated to 0.05μm particles as described above. Beads-lysate, beads-OVA or lysate alone were injected ID. Immunised or naïve C57BL/6 mice were challenged two weeks later intra-peritoneally with 1,000,000 *P. yoelii 17XL* infected RBC.

All the animals survived the challenge, whereas 60% of the animals immunised with the lysate alone (without bead conjugation) failed to control the infection and died (Figure 12). This is the first demonstration of a single dose vaccine being able to confer protection against blood-stage malaria. A single dose vaccine particularly attractive for malaria and other diseases present extensively in the Third world, since it simplifies administration and distribution of the vaccine, ensuring wide population coverage.

Example 12: Cellular Immunity

1) Polysterene beads of 0.05 μm in diameter conjugated to antigens other than OVA, such as the cancer antigen nm23 also induce strong cellular immunity as evidenced by the induction of high levels of IFN gamma secreting T cells (Figure 13).

2) As well as inducing cellular immunity, beads-OVA or beads-nm23 induced high levels of IL4 (Figure 14). This lymphokine promotes the production of antibodies, which may explain why we observe good antibody induction as well as cellular immunity (which requires IFN gamma) was observed.

Example 13: Antibody Immunity

1) Polysterene bead of 0.05 μm in diameter conjugated to OVA induced, after a single intradermal (ID) injection induced high titres of IgG antibody (Figure 15A).

2) Despite high IgG levels, there are no detectable IgE levels. Therefore, this vaccine is demonstrated formally to have no risk

of inducing potentially damaging (since they are involved in allergy) IgE responses (Figure 15B).

Example 14: Administration via different routes and induction of IgG and IgA antibodies

With a view to identifying useful routes of administration of the vaccine in humans, as well as intra-dermally as described above, VSSP conjugated to OVA (100 μg/mouse) was administered to mice intra-peritoneally, sub-cutaneously, intra-nasally and intra-rectally. 3-4 mice per group were tested 30 days after a single immunisation. Similarly to the intra-dermal, all routes induced T cells which secreted IFNg to SIINFEKL or to OVA by ELISPOT assay (1/50,000 to1/2,000 spleen cells). Surprisingly, in contrast to the initial observations using intradermal injection which induced high levels of IgG but little or no IgA, VSSP-OVA by these other routes induced serum IgA responses, and the intra-rectal and intra-nasal route did not induce detectable IgG (titre <1/100) (Table 1). VSSP by the intra-rectal, intra-peritoneal, intra-nasal and subcutaneous routes could therefore also be used to induce protective immunity to diseases where IgA plays a protective role, such as mucosal infections (eg. in lung, cervix or gut).

Table 1

Route	Serum IgG titre	Serum IgA titre
Intra-rectal	<1/100, <1/100, <1/100	1/1280, 1/640, 1/1280
Intra-peritoneal	1/1640, 1/100, 1/400	>1/5120, >1/5120, >1,5120
Intra-nasal	<1/100, <1/100, <1/100, <1/100	1/160, 1/320, 1/320, 1/640
subcutaneous	1/800, 1/200, 1/6560	>1/5120, >1/5120, >1/5120

Unusually high IgG responses after two doses:

Immunisation twice with VSSP-OVA (100 μg/mouse 14 days apart) led to the generation of surprisingly high serum Ig and IgG antibody titres of >1/500,000 as assessed by ELISA. Similar results were obtained for specific IgG antibodies after two immunisations with the breast cancer antigen nm23 and the malaria antigen MSP4/5, when conjugated to VSSP.

Example 15: Long-lasting responses

Responses to VSSP-OVA were surprisingly long-lasting. Figure 16 shows that strong IgG OVA specific antibody by ELISA (Panel A) and CD8 T cell responses to SIINFEKL by IFNg ELISPOT (Panel B) present one year after a single intradermal immunisation (100 μg/mouse). Panel B shows in addition that antigen has to be covalently conjugated to the solid particle for optimal immunogenicity.

Example 16: Heterologous prime-boost

Vaccinia-MUC1 was used to boost responses of animals primed with nothing, peptide cp13- 32 (cp13) from MUC1 in complete Freund's Adjuvant (CFA), Mannan conjugated MUC1 (recombinant MUC1-GST fusion protein)(M-FP) or 0.1 μm VSSP-MUC1 (recombinant MUC1-GST fusion protein)(VSSP). Figure 17 shows responses to the peptide 13-32 region of MUC1 were enhanced in the VSSP-MUC1 primed, Vaccina-MUC1 boosted group compared to animals that received Vaccinia MUC1 alone (Nothing/V compared to VSSP/V). Therefore VSSP-antigen would be suitable for use in heterologous Prime-boost protocols.

Example 17: Material composition of the solid core for VSSP

The inventor's hypothesis that the 0.04-0.05 μm size of solid core is the principal determinant of VSSP immunogenicity predicts that

particles made of material other than polystyrene would be highly immunogenic within this size range. Thus, she compared immunogenicity in mice after a single immunisation intradermally with 0.05μm particles made of polystyrene (PS) or of glass (G1 or G2) and conjugated to OVA using the same chemical procedure (G1) or binding using glutaraldehyde (G2). Figure 18 shows that VSSP made of either polystyrene or glass were similarly highly immunogenic inducing a high precursor frequency of IFNg producing T cells to SIINFEKL by ELISPOT. Therefore, the solid core of VSSP for protein conjugation can be provided by glass as well as polystyrene, and it is anticipated that other materials for the solid core will also be functional, for example PLG.

Example 18: Conjugation of antigen to VSSP

The results show that mixing antigen with 0.05 μm particles makes it more immunogenic than antigen alone, but that covalent linkage is necessary for optimal immunogenicity. The chemical procedure used to conjugate antigen to VSSP could therefore theoretically be a determinant of immunogenicity. Specifically the overall charge of the particle could promote interaction with specific serum or other endogenous proteins. These in turn could theoretically promote uptake by dendritic cells, and cause high immunogenicity. To test for this, mice were immunised with 50nm (i.e. 0.05μm) particles having different charges on the surface. OVA-VSSP has an overall negative charge due to use of carboxylate modified nanoparticles and quenching the activated carboxylic acid groups after conjugation of OVA with glycine (Glycine Figure 19). By quenching the reaction with ethanolamine charges can be neutralised except for the net charge of OVA after conjugation (Alcohol, Figure 19). By quenching with ethylenediamine a positive charge is introduced (Amine, Figure 19). By quenching with aminoacetaldehyde dimethyl acetal (Aldehyde, Figure 19) potentially useful aldehyde groups can be introduced. All three

modifications of the conjugation protocol resulted in highly immunogenic particles, with amine and alcohol modifications being comparable to glycine, and aldehyde slightly less immunogenic. Therefore it is highly unlikely immunogenicity results from non-specific adsorption of serum proteins as the introduction of opposite charges to the particle results in similar immunogenicity. Moreover, some alternative modifications to the conjugation procedure, and changes in charges can be introduced with no decrease in immunogenicity.

DISCUSSION

In view of the results above, the composition of the invention provides a way to further improve or optimise vaccines or vaccination strategy that could apply to a variety of infections, cancer or other diseases.

The optimal size of the VSSP coincides with that of most known viruses (30-150nm). Hence, it is tempting to speculate that use of the VSSPs is biologically significant. From the above observations, the inventor believes that the immune system may be geared to react fully to particles of the size range of the VSSPs. Before the present invention, it was not known or understood that the stimulation of an immune response could depend to a great extent on the size of an immune stimulant that falls within the size range of viruses, especially when epitopes from other pathogens eg bacteria, fungi are considerably large. Indeed, antigens targeted through this pathway elicited surprisingly broad (comprising both humoral and cellular arms of the immune response) and strong responses (inducing rapidly long lasting high effector T cell precursor frequencies) suggesting the immune system may be geared to react fully to particles of viral size. Previous studies utilising VLPs (i.e. pure antigen not linked to a particle) comprised of HepB surface proteins, or the yeast retrotransposon protein (Ty) have also shown broad and long lasting immunity induced by a single immunizing dose, although responses were still 10-100 fold

lower than with VSSPs [1, 2, 3, 19, 20]. However it has been assumed that characteristics other than size alone, such as their lipid or mannan content, or membrane biding proteins are responsible for the ability of VLPs to induce class I restricted T cell responses [1]. Not wishing to be bound by theory, the inventor considers that the combination of efficient targeting to antigen presenting cells such as dendritic cells *in vivo* by VSSPs, followed by potential slow antigen release by proteolysis from VSSPs, may have generated particularly powerful immunogens.

Use of VSSP as novel vaccines was demonstrated by the ability of a single immunising dose to protect against subsequent challenge with tumor cells in the OVA model. The inventor has also observed broad and strong immunogenicity and protection to an antigen expressed in breast cancer, mucin-1 (MUC-1). The intradermal route of administration utilised in their animal studies may be easily implemented in humans. VSSPs may thus offer a particularly attractive and simple strategy for human vaccine development, in particular to diseases where both humoral and cellular immunity participate in generating protection, such as malaria, cancer and viral diseases, notably, AIDS and hepatitis [10, 12, 21, 25-28]. The targeting of recombinant antigen to class I presentation pathway also offers the possibility of inducing T cell responses to multiple epitopes, and thus would extend the use of such vaccines in a MHC diverse target human population.

At present, priming of DCs for effective stimulation of CTLs is by *ex vivo* pulsing of DCs but this is expensive and logistically difficult. The present invention provides an alternative to efficiently deliver antigens to DCs *in vivo*, leading to the subsequent induction of high numbers of antigen specific CD8 T-cells and immune protection. The ability of the VSSPs within the narrow size range of 0.04-0.05μm to induce singularly high CD8 T-cell levels could be the consequence of efficient uptake by APCs or by a potent subset, targeting to the MHC 1 processing pathway and/or direct stimulation of APC function. Uptake

of the VSSPs was found to be enhanced in the lymph node, compared to other sizes, and this enhancement was attributed to increased frequencies of particle positive DEC205+ cells, a marker of DCs. DCs are powerful APC and expression of CD40 and CD86 further characterises a subset capable of efficient CD8 T-cell priming. These markers were found in a high proportion of VSSP+ cells. Thus, uptake and selective localisation of VSSP in this potent DC subset *in vivo* could explain the immunogenicity of the microparticles according to the invention.

Other advantages of the VSSP of the invention include the ability to induce immune responses including IgA production following administration *via* a number of routes, and their suitability for prime-boost vaccination strategy.

Further studies will involve the use of the VSSPs to determine the physiological mechanisms that make them elicit the unique immune response obtained.

It is to be understood that various other embodiments of the invention that is described herein may be made by those skilled in the art without departing from the central concept underlying the invention.

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CLAIMS:-

1. An immunogenic composition comprising at least one antigen in association with microparticles, wherein the microparticles are in the same size range as viruses.

2. A vaccine composition comprising at least one antigen in association with microparticles, wherein the microparticles are in the same size range as viruses.

3. The composition of Claim 1 or Claim 2 wherein the microparticles are of substantially uniform size.

4. The composition of any one of Claims 1 to 3 wherein said microparticles comprise a solid core.

5. The composition of any one of Claims 1 to 4 wherein said microparticles are about 0.03 to 0.05μm in diameter.

6. The composition of any one of Claims 1 to 5 wherein said microparticles are made from latex, ferrous molecules, gold, glass, calcium phosphate, polystyrene, poly lyseine G or other biodegradable and biocompatible polymers.

7. The composition of any one of Claims 1 to 6 wherein said antigen is adsorbed onto, conjugated to, or covalently coupled to said microparticles.

8. The composition of any one of Claims 1 to 7 wherein said antigen is a peptide, protein, recombinant peptide or protein, lipid, carbohydrate, nucleic acid or other type of molecule or a combination of any of these.

9. The composition of any one of Claims 1 to 8 wherein the antigen is derived from a pathogen, tissue, cell organ or molecule and is selected from the following group:-

pollen, hepatitis C virus, (HIV) core, E1, E2 and NS2 proteins, antigens from Plasmodium species such as P. vivax and other Plasmodium species including P. faliciparum circumsporozoite protein (CS) and human plasmodium falciparum, -vivax, -ovalae and malariae, TRAP, MSP-1, MSP-2, MSP-3, MSP-4, MSP-5, AMA-1, RESA,

SALSA, STARP, LSA1 and LSA3, HIV-gp120/160 envelope glycoprotein, streptococcus surface protein Ag, influenza nucleoprotein, haemagglutinin-neuraminidase surface infection, TcpA pilin subunit, VP1 protein, LMCV nucleoprotein, Leishmania major surface glycoprotein (gp63), Bordetella pertussis surface protein, rabies virus G protein, Streptococcus M protein, Staphylococcal proteins or Helicobacter pylori proteins, Syncyticial virus (RSV) F or G proteins, Epstein Barr virus (EBV) gp340 or nucleoantigen 3A, haemagglutinin, Borrelia burgdorferi outer surface protein (Osp) A, Mycobacterium tuberculosis 38kD lipoprotein or 30kD protein (Ag85), 10kD or 65kD proteins, Neisseria meningitidis class 1 outer protein, Varicella zoster virus IE62 and gpl, Rubella virus capsid protein, Hepatitis B virus pre S1 ag, Herpes simplex virus type I glycoprotein G or gp D or CP27, Murray valley encephalitis virus E glycoprotein, Hepatitis A virus VP1, polio virus capsid protein VP1, VP2 and VP3, chlamydia trachomatis surface protein, Hepatitis B virus envelope Ag pre S2, Human rhinovirus (HRV) capsid, papillomavirus peptides from oncogene E6 and E7, Listeria surface protein, Varicella virus envelope protein, Vaccinia virus envelope protein, Brucella surface protein, Rotavirus, VP-3, VP-4, VP-5, VP-7 and VP–8, a combination of one or more of said antigens, an amino acid subunit of said antigen comprising five or more amino acids in length or combinations of one or more of said subunits.

10. The composition of any one of Claims 1 to 8 wherein the antigen is derived from a cancer such as a breast cancer, lung cancer, pancreas cancer, colon cancer or melanoma.

11. The composition of Claim 10, wherein the antigen is a recombinant peptide or protein.

12. A method of eliciting an immune response in a subject said method comprising administering to a subject an immunologically effective amount of a composition comprising at least one antigen associated with microparticles, wherein the microparticles are in the same size range as viruses.

13. The method of Claim 11 wherein the immune response is selected from the group consisting of a CD8 cellular response and an antibody response wherein the antibody is IgG, IgM or IgA.

14. The method of Claim 11 wherein said immune response is the proliferation and/or expansion of dendritic cells.

15. A method of eliciting a protective immune response to an antigen via a single dose said method comprising administering, once only to a subject, an immunologically effective amount of a composition comprising at least one antigen associated with microparticles, wherein the microparticles are in the same size range as viruses and the immune response comprises the stimulation and/or proliferation of dendritic cells.

16. A method of <u>in vivo</u> delivery of an antigen to dendritic cells in order to elicit an immune response said method comprising administering to a subject an immunologically effective amount of a composition comprising at least one antigen associated with microparticles, wherein the microparticles are in the same size range as viruses.

17. The method of Claim 15 or Claim 16 wherein the immune response comprises eliciting mechanisms for MHC class I presentation of the antigen which antigen is taken up by caveole and/or clathrin pits for further processing by Rab4 independent and TAP dependent processes.

18. A method of producing an immunogenic microparticle/antigen composition comprising contacting microparticles which are in the same size range as viruses with one or more antigens such that the microparticles and antigens become associated.

FIGURE 1

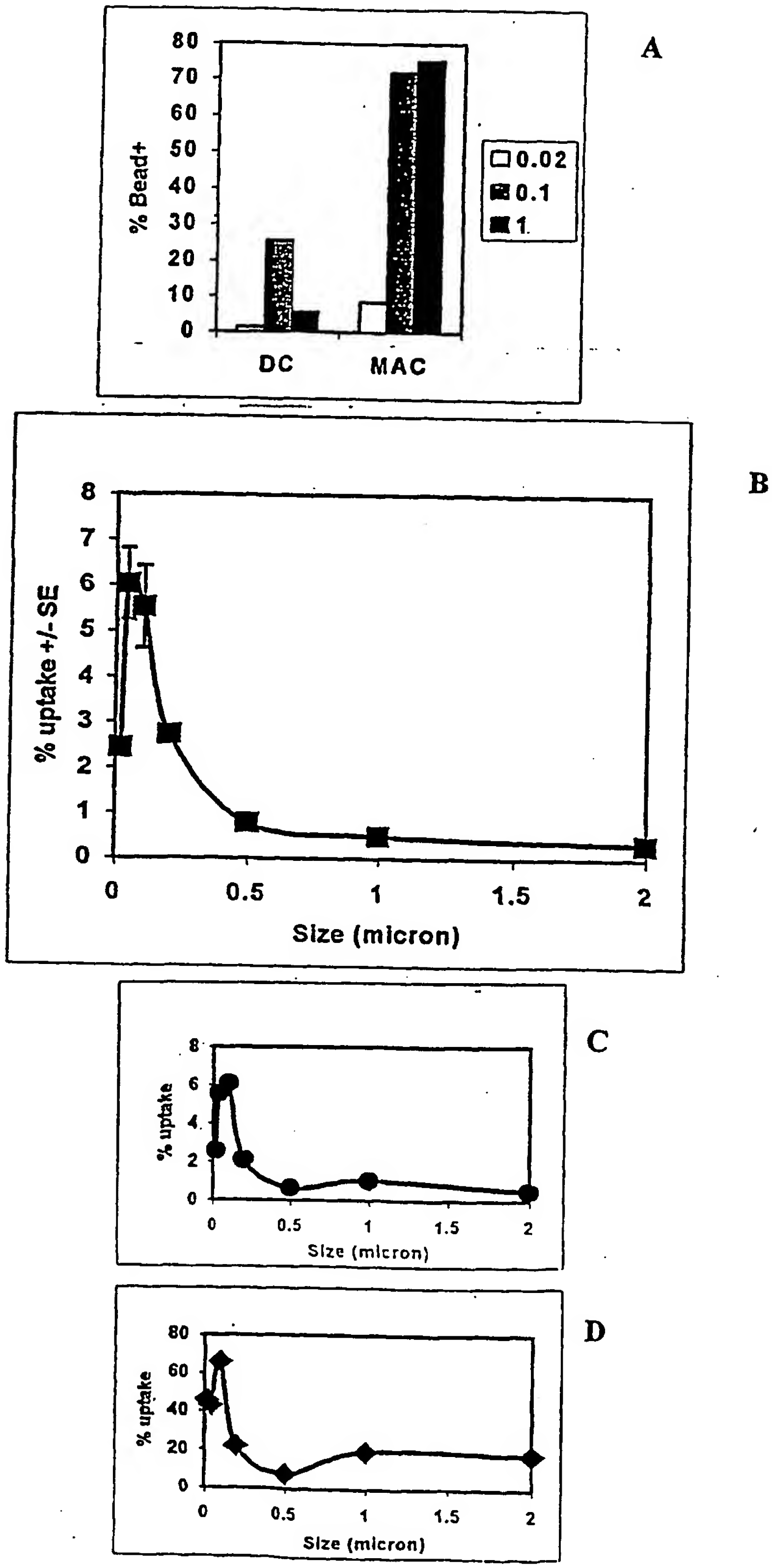
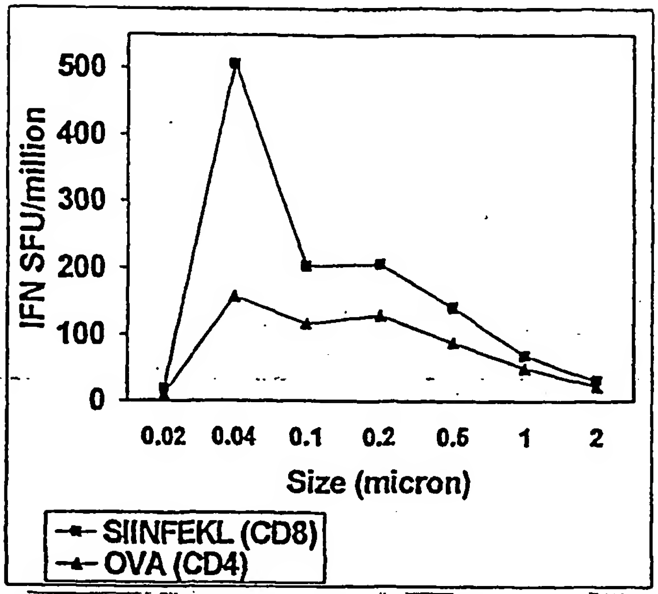
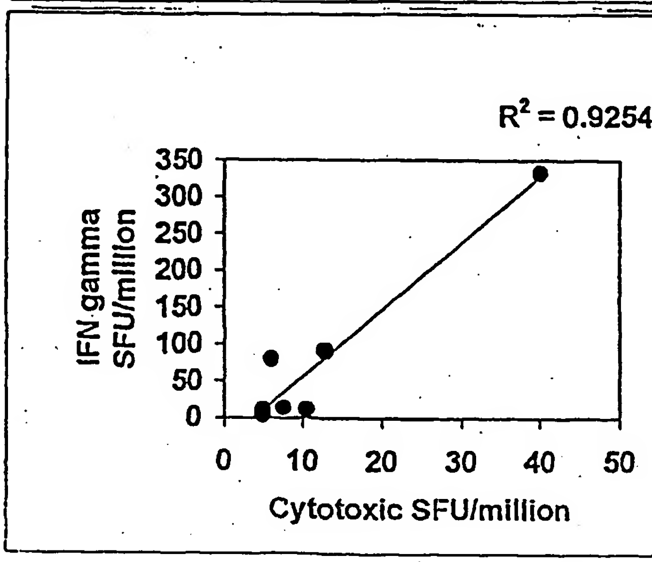

A
% Bead+
80
70
60
50
40
30
20
10
0
DC
MAC
0.02
0.1
1
B
% uptake +/- SE
8
7
6
5
4
3
2
1
0
0
0.5
1
1.5
2
Size (micron)
C
% uptake
8
6
4
2
0
0
0.5
1
1.5
2
Size (micron)
D
% uptake
80
60
40
20
0
0
0.5
1
1.5
2
Size (micron)

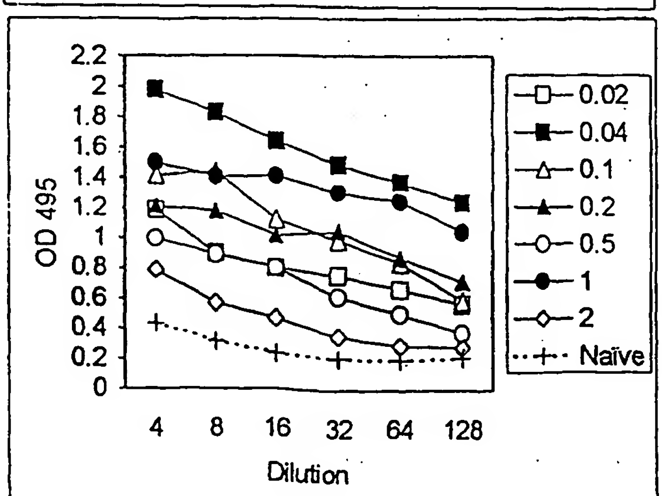
FIGURE 2


500
400
300
200
100
0
IFN SFU/million
0.02 0.04 0.1 0.2 0.5 1 2
Size (micron)
SIINFEKL (CD8)
OVA (CD4)

A

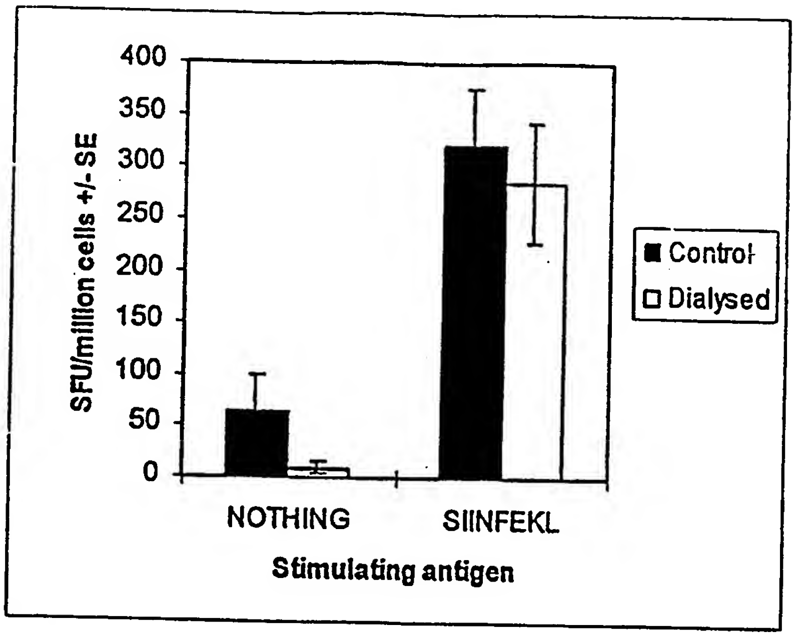

R² = 0.9254
350
300
250
200
150
100
50
0
IFN gamma SFU/million
0 10 20 30 40 50
Cytotoxic SFU/million

B

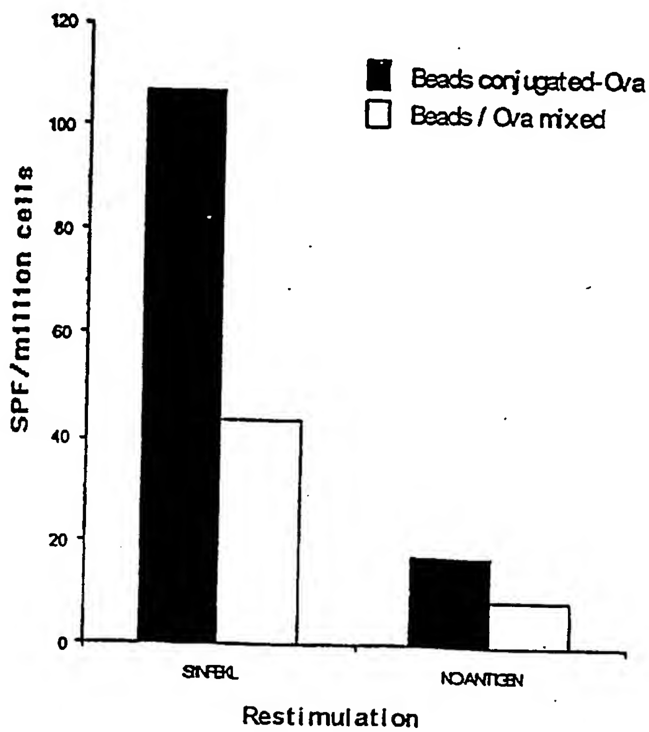

2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD 495
4 8 16 32 64 128
Dilution
0.02
0.04
0.1
0.2
0.5
1
2
Naïve

C

FIGURE 3


400
350
300
250
200
150
100
50
0
SFU/million cells +/- SE
Control
Dialysed
NOTHING
SIINFEKL
Stimulating antigen

A


120
100
80
60
40
20
0
SPF/million cells
Beads conjugated-Ova
Beads / Ova mixed
Restimulation

B

FIGURE 4

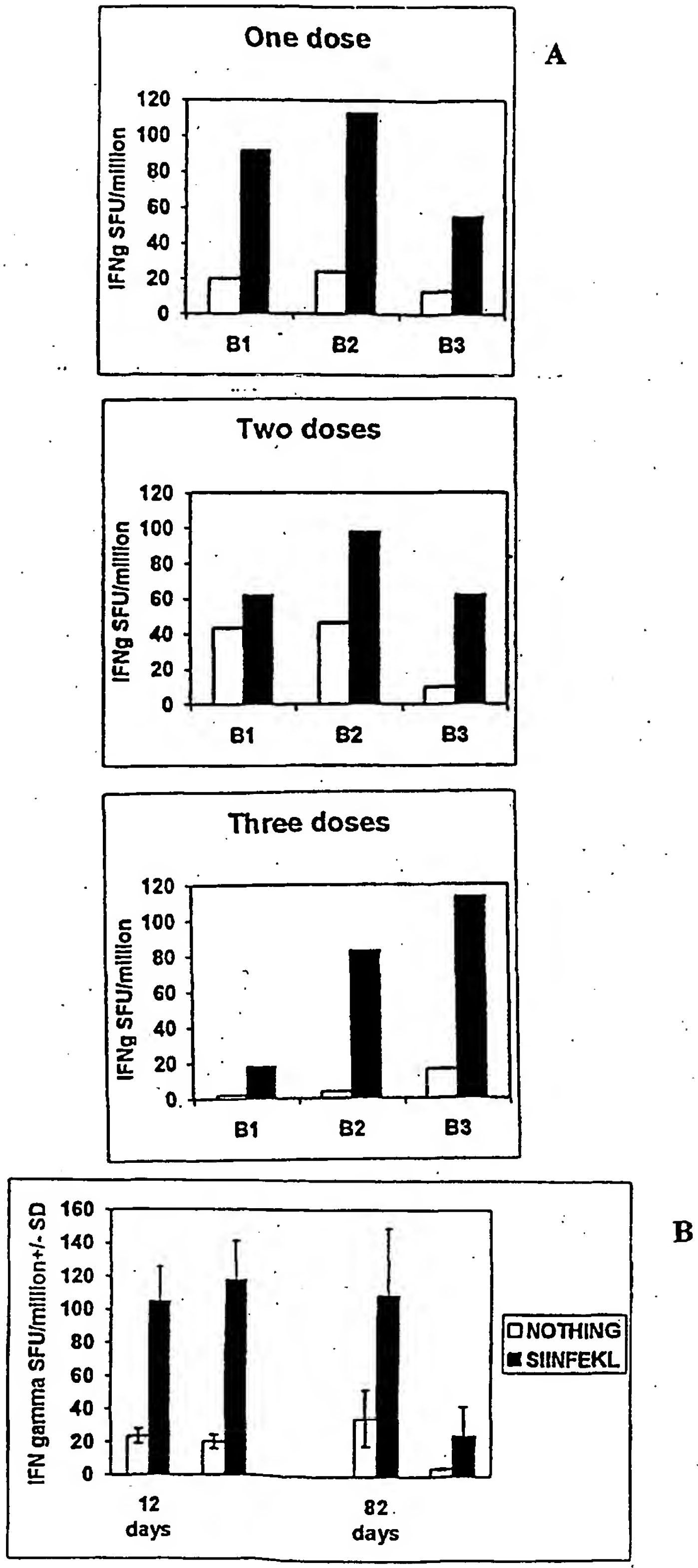

One dose
A
IFNg SFU/million
120
100
80
60
40
20
0
B1
B2
B3
Two doses
IFNg SFU/million
120
100
80
60
40
20
0
B1
B2
B3
Three doses
IFNg SFU/million
120
100
80
60
40
20
0
B1
B2
B3
B
IFN gamma SFU/million+/- SD
160
140
120
100
80
60
40
20
0
12 days
82 days
NOTHING
SIINFEKL

FIGURE 5

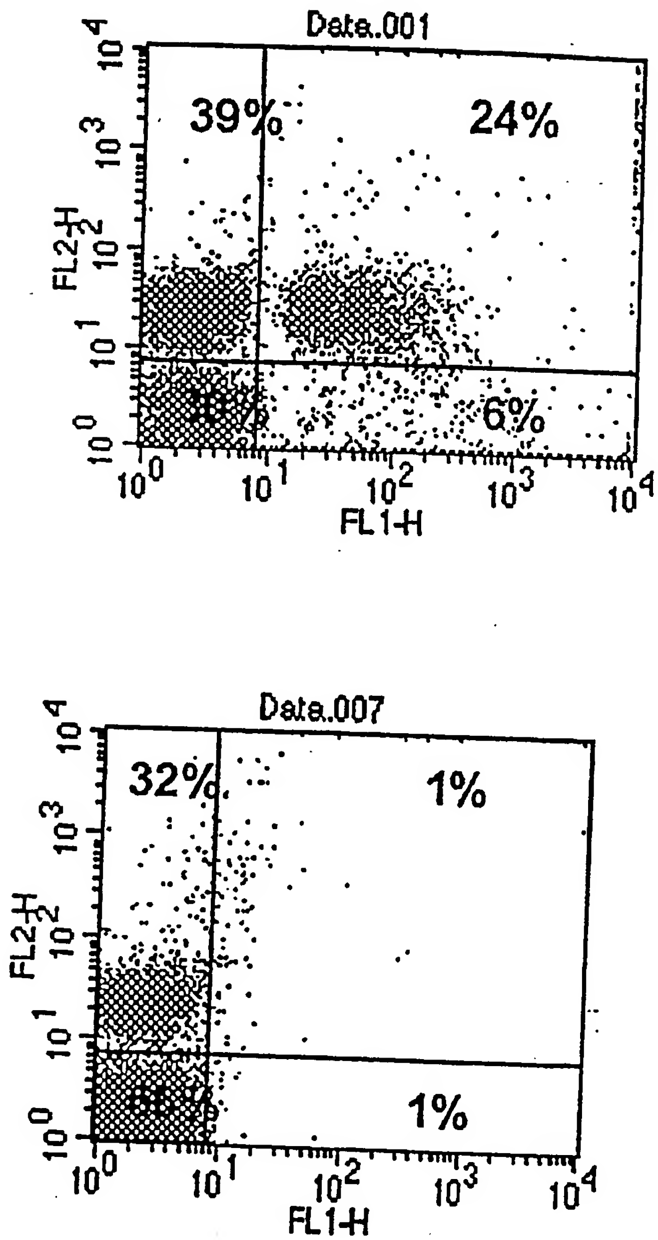


FIGURE 6

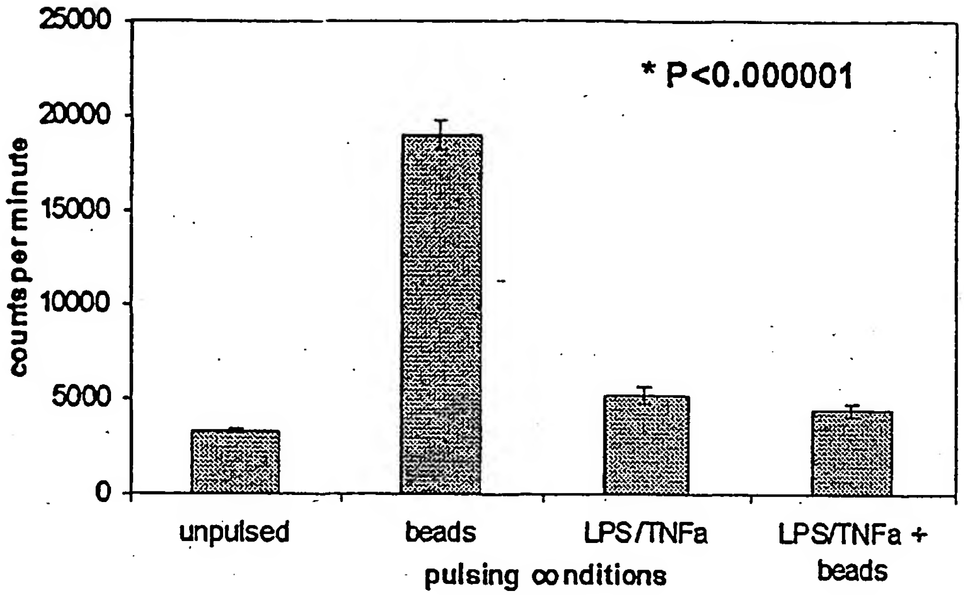

* P<0.000001
counts per minute
25000
20000
15000
10000
5000
0
unpulsed
beads
LPS/TNFa
LPS/TNFa + beads
pulsing conditions

FIGURE 7

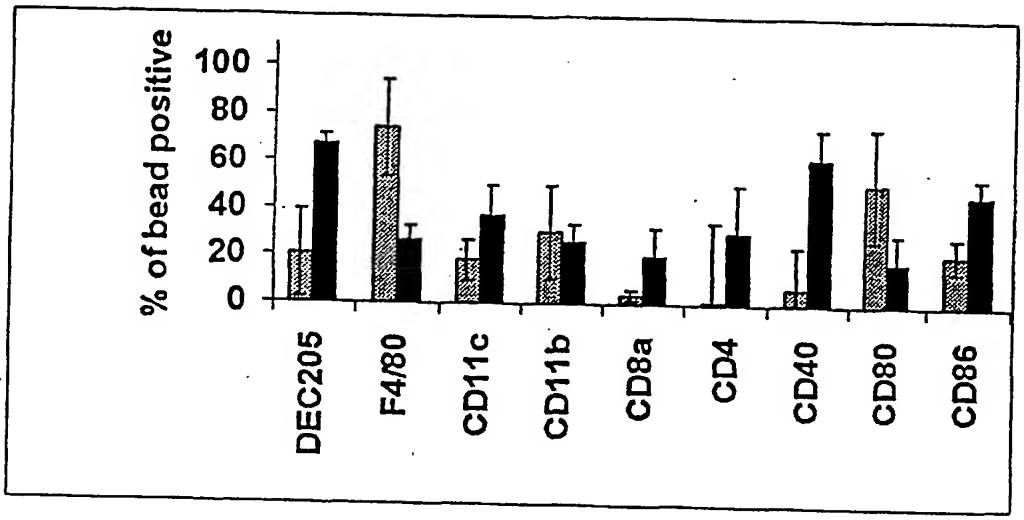

% of bead positive
100
80
60
40
20
0
DEC205
F4/80
CD11c
CD11b
CD8a
CD4
CD40
CD80
CD86

FIGURE 8A

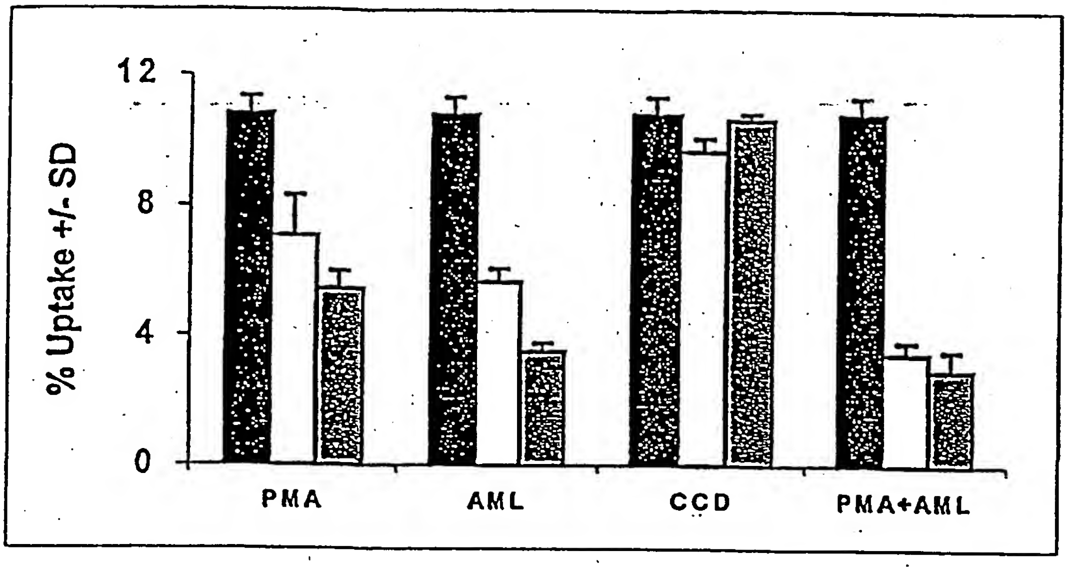
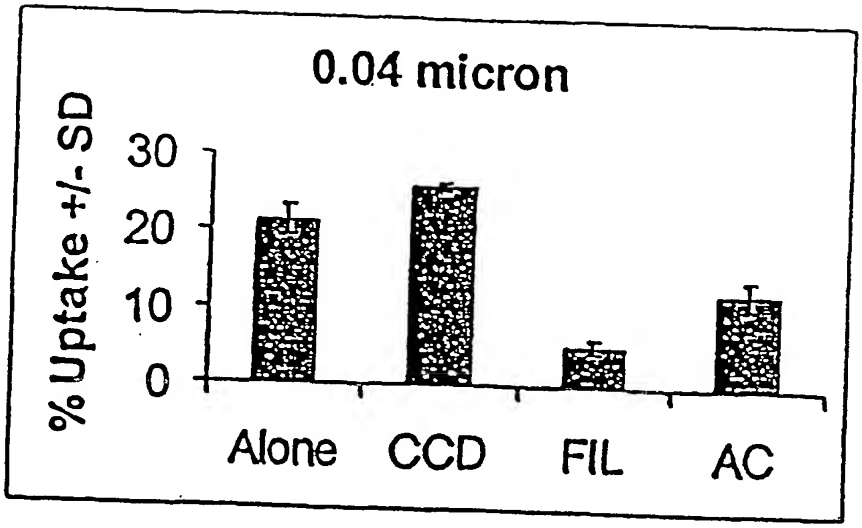


FIGURE 8B


0.04 micron
% Uptake +/- SD
30
20
10
0
Alone
CCD
FIL
AC

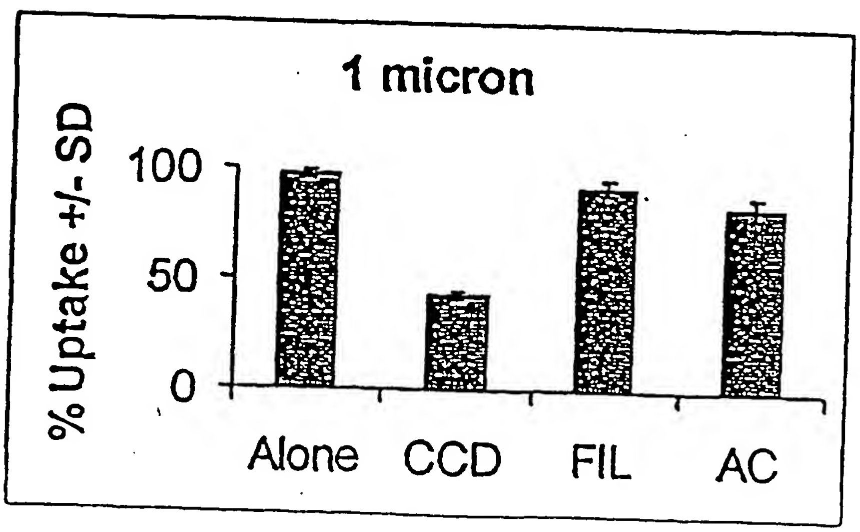

1 micron
% Uptake +/- SD
100
50
0
Alone
CCD
FIL
AC

FIGURE 9

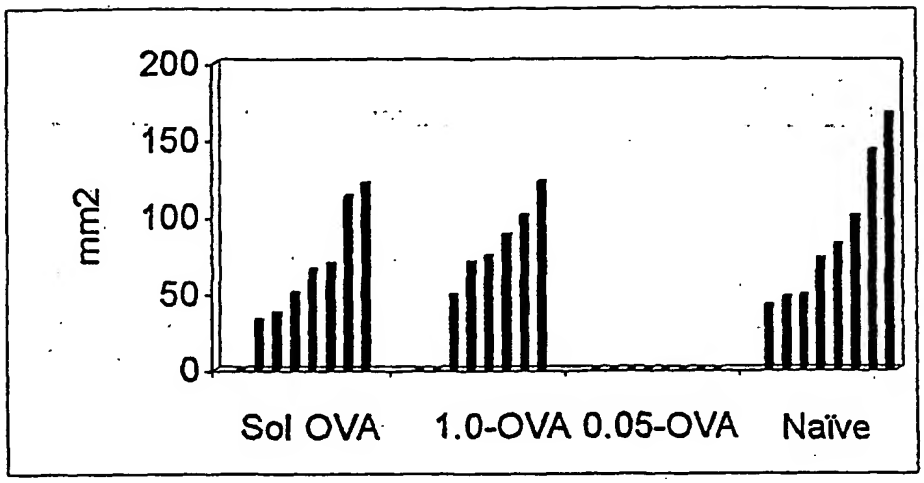


FIGURE 10

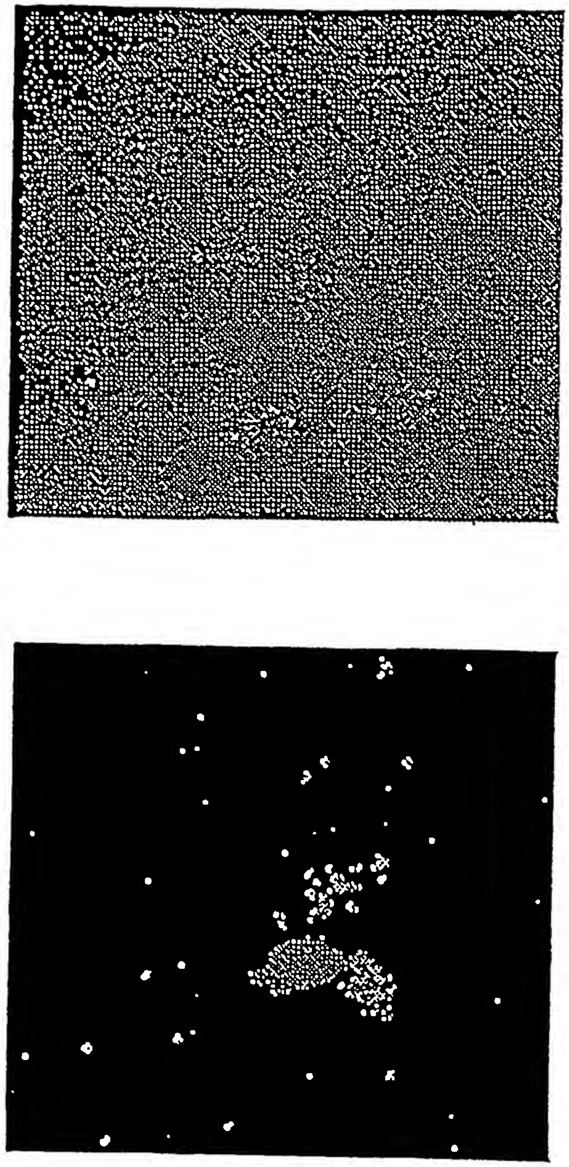


FIGURE 11

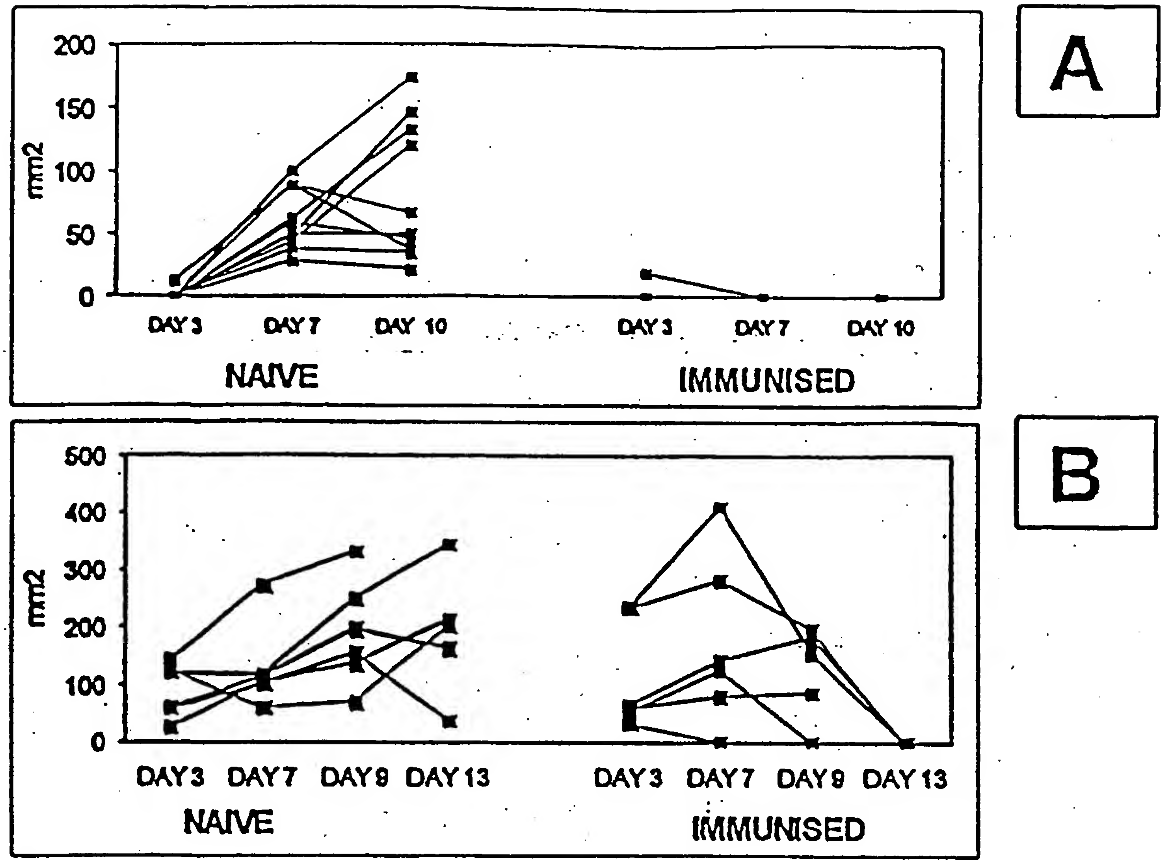

A
200
150
100
50
0
mm2
DAY 3
DAY 7
DAY 10
NAIVE
DAY 3
DAY 7
DAY 10
IMMUNISED
B
500
400
300
200
100
0
mm2
DAY3
DAY7
DAY9
DAY13
NAIVE
DAY3
DAY7
DAY9
DAY13
IMMUNISED

FIGURE 12

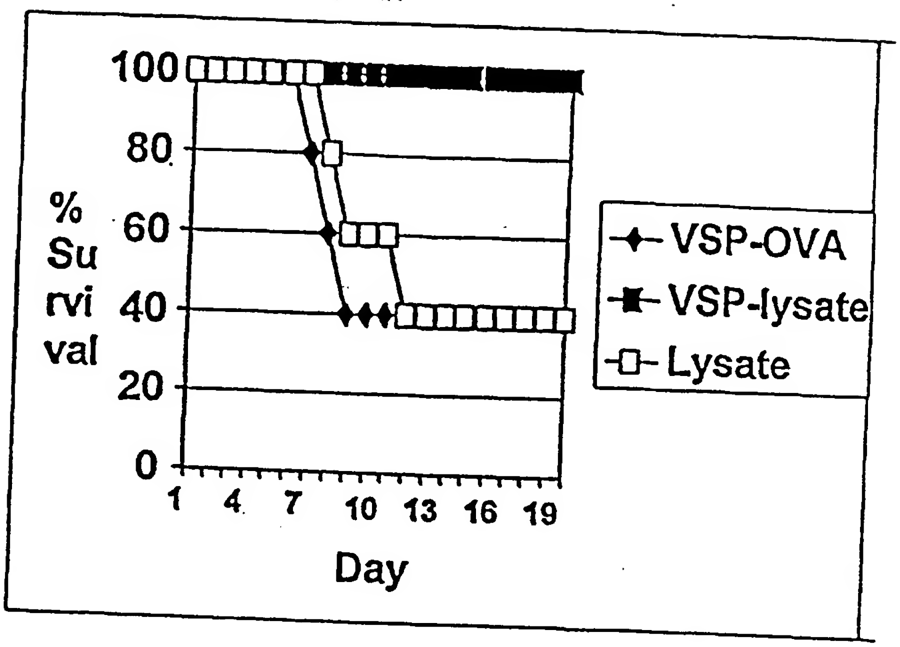

100
80
60
40
20
0
% Survival
1
4
7
10
13
16
19
Day
VSP-OVA
VSP-lysate
Lysate

FIGURE 13

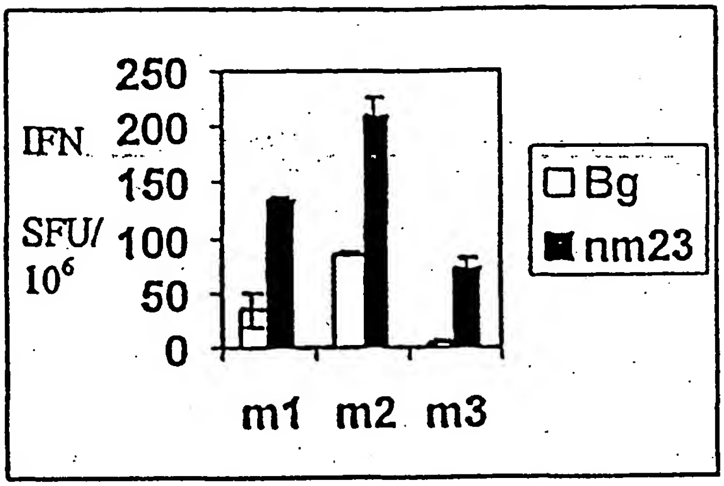


FIGURE 14

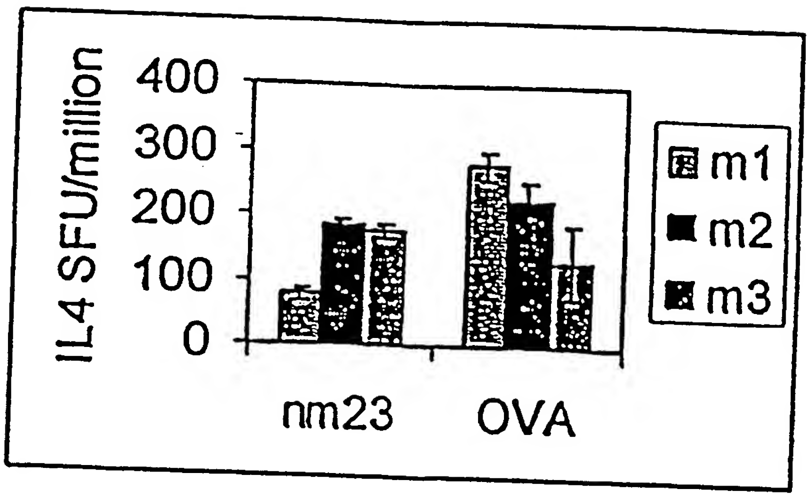


FIGURE 15A

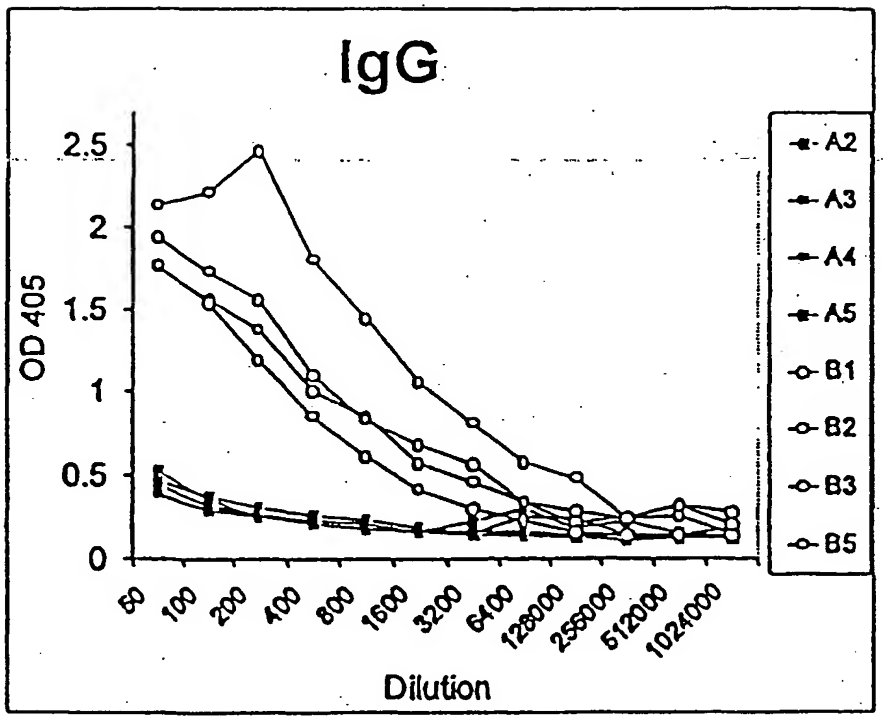

IgG
OD 405
2.5
2
1.5
1
0.5
0
50
100
200
400
800
1600
3200
6400
128000
256000
512000
1024000
Dilution
A2
A3
A4
A5
B1
B2
B3
B5

FIGURE 15B

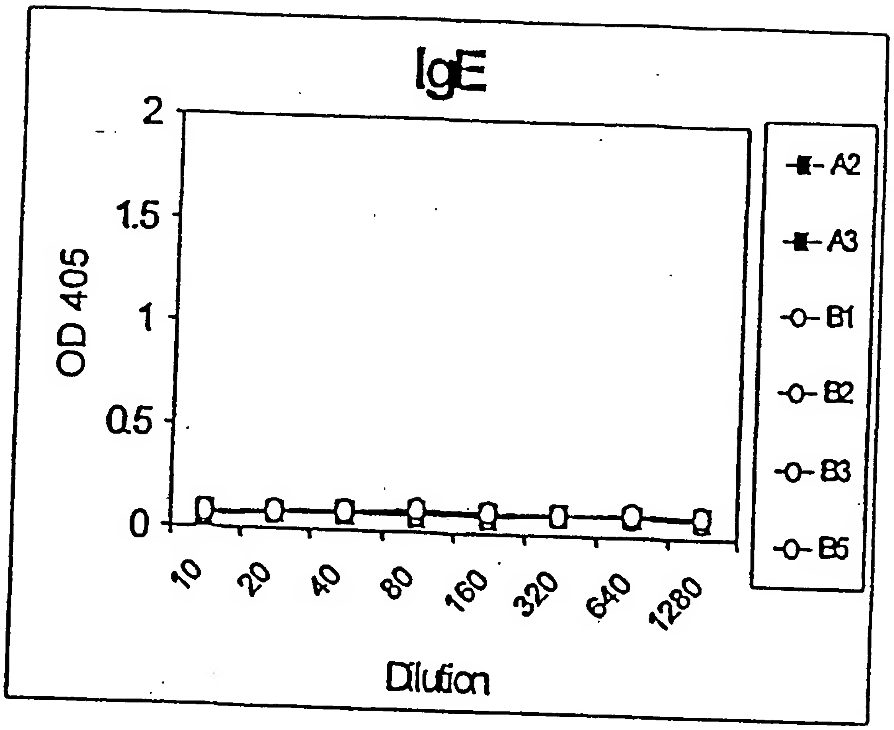
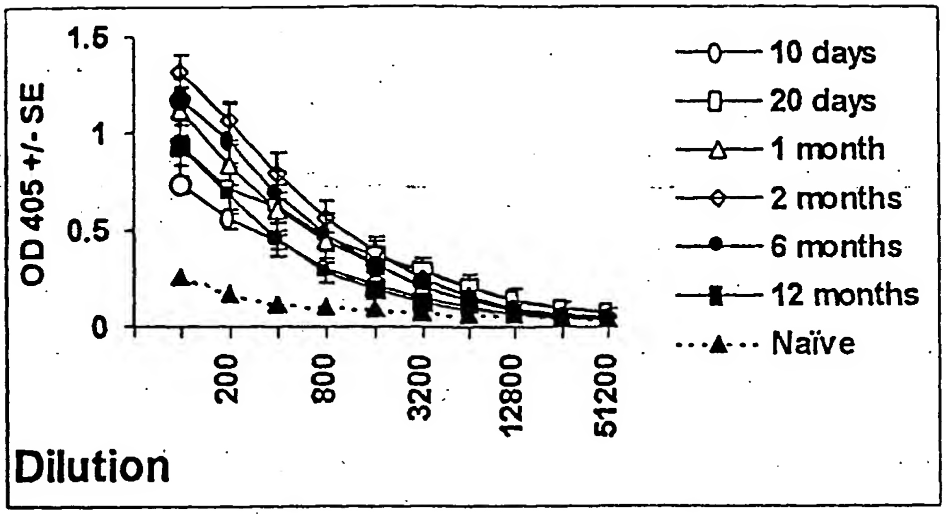
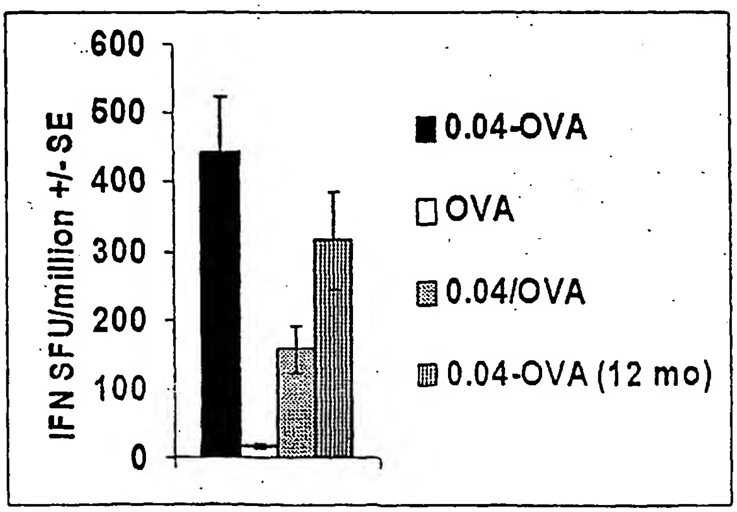

IgE
OD 405
2
1.5
1
0.5
0
10
20
40
80
160
320
640
1280
Dilution
A2
A3
B1
B2
B3
B5

FIGURE 16


1.5
1
0.5
0
OD 405 +/- SE
200
800
3200
12800
51200
Dilution
10 days
20 days
1 month
2 months
6 months
12 months
Naïve

A


600
500
400
300
200
100
0
IFN SFU/million +/- SE
0.04-OVA
OVA
0.04/OVA
0.04-OVA (12 mo)

B

FIGURE 17

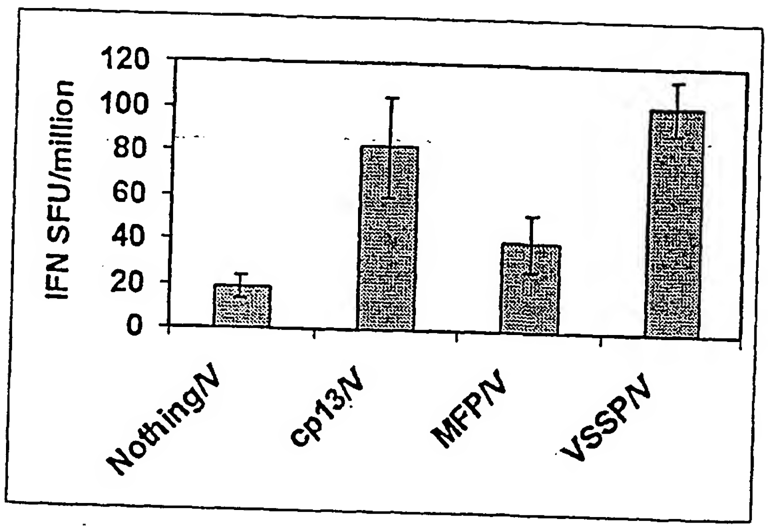

IFN SFU/million
120
100
80
60
40
20
0
Nothing/V
cp13/V
MFP/V
VSSP/V

FIGURE 18

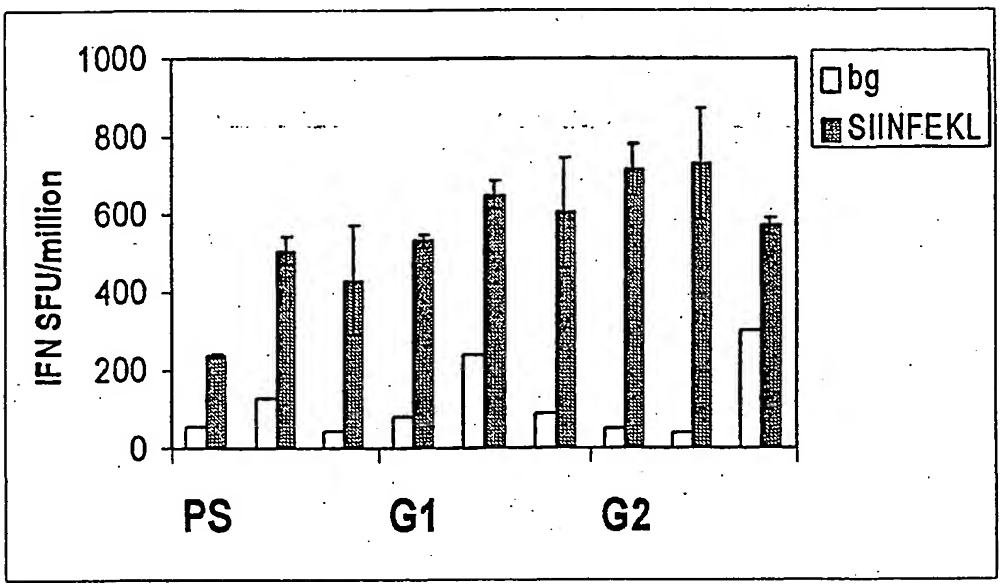
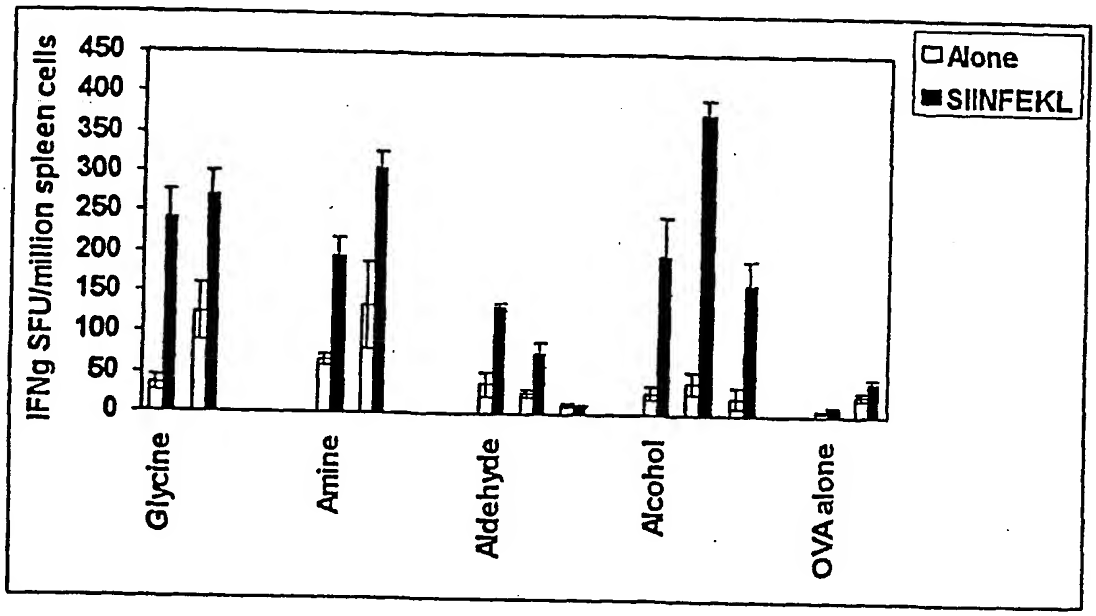


FIGURE 19


IFNg SFU/million spleen cells
450
400
350
300
250
200
150
100
50
0
Alone
SIINFEKL
Glycine
Amine
Aldehyde
Alcohol
OVA alone

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/01160

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. 7: A61K 39/00, 47/00, 47/30; A61P 37/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Refer Electronic database consulted below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT: A61K-047/IC & Keywords: antigen, immunogen, size, dendrite and similar terms;
MEDLINE & Keywords: microsphere, nanosphere, size, diameter, antigen, immunogen, dendrite and similar terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	US 4225581 A (KREUTER et. al.) 30 September 1980 (cited in the present application)	
X	See column 4, examples and claims	1-18
	WO 91/16072 A (PRESIDENT AND FELLOWS OF HARVARD COLLEGE; INSTITUT SUISSE DE RECHERCHES EXPERIMENTALES SUR LE CANCER) 31 October 1991	
X	See page 7, examples and claims	1-18
	US 5178882 A (KOSSOVSKY et. al.) 12 January 1993	
X	See examples and claims	1-18

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 November 2001	15 NOV 2001

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	KAREN TAN Telephone No : (02) 6283 2277

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/01160

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	TOBIO, M. et. al. Stealth PLA-PEG nanoparticles as protein carriers for nasal administration. Pharmaceutical Research. 1998, vol. 15 no. 2, pages 270-275	
X	See whole document	1-18
	WO 98/01161 A (DANBIOSYST UK LIMITED) 15 January 1998	
X	See pages 6, 12, examples and claims	1-18
	TABATA, Y. et. al. Size effect on systemic and mucosal immune responses induced by oral administration of biodegradable microspheres. Vaccine. 1996, vol. 14, no. 17-18, pages 1677-1685	
X	See whole document	1-18
	DESAI, M, P. et. al. Immune response with biodegradable nanospheres and alum: studies in rabbits using staphylococcal enterotoxinB-toxoid. Journal of Microencapsulation. March-April 2000, vol. 17 no. 2, pages 215-225	
X	See whole document	1-18
	BENNS, J. M. and Kim, S. W. Tailoring new gene delivery designs for specific targets, Journal of Drug Targeting. 2000, vol. 8 no. 1, pages 1-12	
X	See whole document	1-18
	AU 31613/01 A (CNRS CENT NAT RECH SCI; IDM IMMUNO-DESIGNED MOLECULES; INSERM INST NAT SANTE & RECH MEDICALE)	
P, X	See page 10, examples and claims	1-18
	THIELE, L. et. al. Evaluation of particle uptake in human blood monocyte-derived cells in vitro. Does phagocytosis activity of dendritic cells measure up with macrophages? Journal of Controlled Release. 2001, vol. 76, pages 59-71	
P, X	See whole document	1-18

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU01/01160

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	4225581	AU	12208/76	BE	839748	CH	614856
		DE	2611143	DK	1050/76	FR	2304326
		GB	1544107	JP	51118823	NL	7602717
		US	4269821	ZA	7601695	CH	618352
WO	9116072	AU	76849/91	BR	9105718	CA	2060318
		EP	477339	HU	63337	NO	914945
		US	5443832	ZA	9102838		
US	5178882	AU	79210/91	CA	2045204	EP	465081
		IE	912150	JP	5255111	PT	98066
		US	5219577	US	5334394	US	5462750
		US	5306508	US	5441739	US	5460830
		US	5460831	US	5462751	US	5464634
		US	5639505	AU	56009/94	AU	57266/94
		AU	69554/94	CA	2152379	CA	2152490
		CA	2153147	EP	676954	EP	676955
		EP	689421	WO	9415581	WO	9415585
		WO	9415586	CA	2174244	EP	726767
		WO	9512392	AU	23616/95	EP	758226
		WO	9528915				
WO	9801161	AU	34545/97	AU	34546/97	CA	2257300
		EP	986404	EP	994726	GB	2330532
		GB	2330534	NO	990002	NO	990003
		WO	9801160				
AU	31613/01	WO	0152884	EP	1118331		

END OF ANNEX

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